# A Novel Procedure for Qualifying and Quantifying Mono-, Di-, and Trisaccharides by Derivatization and Gas Chromatography

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for Graduation

*With Research Distinction* in the Undergraduate Colleges

of The Ohio State University

By

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June 2011

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# Acknowledgement

I would like to thank my research advisor, Dr. Christopher Callam, for giving me these projects. His trust and encouragements fostered my independence, work ethics, and interest in research. Also, I would like to thank my coworker, Michael Woodruff, for his guidance, which facilitated my transition into working independently and helped me to reach deeper understandings of my own work. Furthermore, I appreciate Dr. QiQi Zhou from The Ohio State Medical Center and Dr. Samantha King from Nationwide Children's Hospital for providing wonderful opportunities to apply my method to urine analysis and the study of cellular permeability. Last but not least, I would like to thank the Department of Chemistry for their support.

# Abstract

The identification and quantification of carbohydrate content in aqueous solutions and biological systems are of great interest and importance. The polar nature and large sizes of sugars can cause difficulties in identification and separation processes. Problems include long retention times and degradation during separation procedures, such as column chromatography or gas chromatography (GC). Previous work in GC analysis of carbohydrates has shown quantitative results with high levels of noise due to decomposition and relatively poor detection limits. This project has developed methodology for an optimal quantification and identification procedure. We have derivatized carbohydrates to improve detection limits and resolution in standard GC and gas chromatography and mass spectrometry (GC/MS) analysis. This method has allowed for the identification and the separation of a synthetic mixture of 18 compounds, the qualitative identification and quantification of trace carbohydrate content in rat urine, and the analysis of cellular membrane permeability of a disaccharide. The necessary steps of this procedure involve converting carbonyl groups (aldehyde or ketone) into oximes, protection of the alcohols as trimethylsilyl ethers, concentration, and non-polar extraction. This study has the potential to make identification and quantification in biochemistry and medicinal chemistry less time-consuming and more accurate.

# Introduction

Non-metabolizable markers, such as polyethylene glycol, sugars, and radiolabeled molecules, are especially important to permeability studies within biological systems[7]. Sugars used as biomarkers or probes are of particular interest to biological chemists because they are much easier and safer to use in live animal models or humans. Semi-permeable biological barriers, such as the intestinal mucosal epithelium, the glomeruli within kidneys, and the plasma membrane, serve important roles in nutrient and drug absorption, and metabolite and waste elimination. For example, non-endogenously-produced monosaccharides and disaccharides are commonly used to analyze gastrointestinal (GI) permeability and absorption capabilities in normal and damaged intestine, such as in alcoholics. It is important to understand that various sugars are absorbed at different capacities in specific segments of the gastrointestinal tract. For instance, whereas sucrose is decomposed upon entering the duodenum (the first segment of the small intestines), lactulose and mannitol are only metabolized by commensal flora (normally bacteria) within the ileum (the distal portion of the small intestine)[7] and the colon. In comparison, the recently-discovered sweetener, sucralose, is absorbed passively across small and large intestine, into the blood, and excreted unaltered in the urine[1]. The intestinal permeability to these markers mentioned previously tends to increase in damaged intestinal mucosa, thus these sugars are excreted more readily in the urine. Other than analyzing gastrointestinal damage, sugars are also a safe and convenient way to measure the degree of diabetic nephropathy, kidney damage caused by diabetes[9]. While existing laboratory tests, such

as quantifying blood urea nitrogen (BUN) or creatinine, are sufficient in diagnosing the presence of nephropathy, they are not accurate due to the endogenous nature of the analyte. An intravenous injection of non-endogenous and non-metabolizable sugars coupled with the quantification of the sugars in urine can be a useful way to accurately analyze the degree of glomerular damage. Lastly, it is also important to know the cells absorptive capacity of saccharides with therapeutic potentials. Thus, sugars are greatly beneficial for the analysis of multiple organ systems at multiple levels of organization. Recently, determining the ratio and the concentration of these compounds is an important focal point of biological and analytical chemistr, however this remains a difficult analytical challenge today.

Gas chromatography is a widely used method for the separation of organic compounds. A GC system uses a carrier gas or a combination of gases, such as helium or nitrogen, to mobilize the vaporized compound(s) through a column and eventually to a detector. The signals from the detector are then recorded on a computer. The column is situated in an oven, which has controlled temperature change to ensure the mobilization of vapors. This column is typically a long and thin tubular coil that is coated with polar or non-polar materials, such as phenyl methyl siloxane, on the inner surface. The coating material can interact with particular molecules, delaying their flight to various degrees, and in turn separating them on the resulting chromatograph recorded on a computer. The *x-axis* of a gas chromatograph represents the retention time – the time it takes for a particular molecule to interact with the coating material, travel the whole length of the coiled column, and reach the detector. Its *y-axis* represents the magnitude of the signal. On a resulting chromatograph, each substance would be

separated and appear as a peak. The retention time is typically used to identify the molecule. The area underneath each peak is proportional to the quantity of a particular molecule, assuming equal interaction with the detector. The qualification and quantification are possible knowing the retention time and the area of a certain molecule respectively.

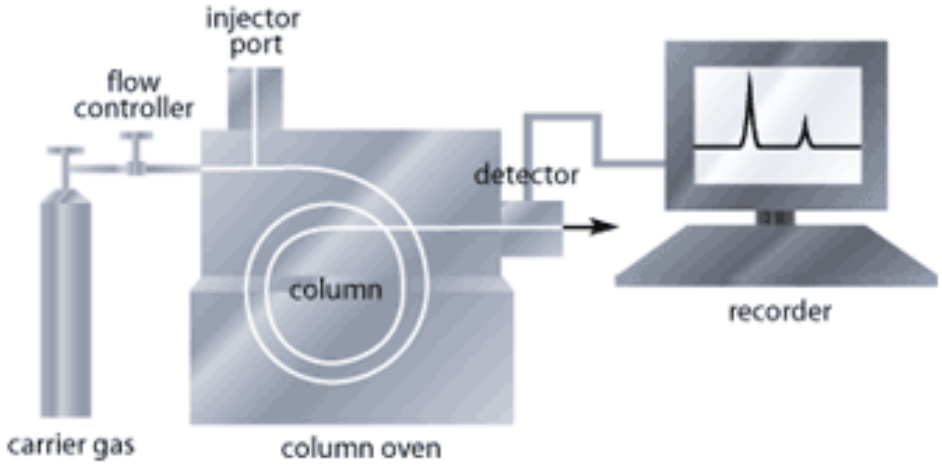
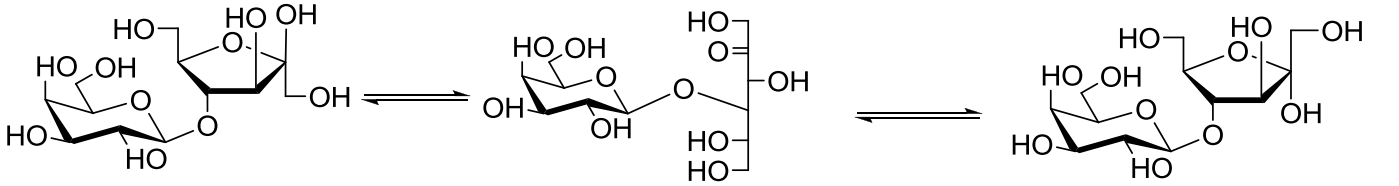



Figure 1: Simplified schematic diagram of a gas chromatograph. The carrier gas flows into the column at a controlled rate, picks up chemicals from the automatic injector port, and transports them to the detector. The detector discussed here is a flame ionization detector (FID) or a mass quadrapole detector (MS). The signals detected are sent to a recording device, in this case, a computer[2].

This thesis focuses on analyzing a variety of carbohydrates ranging from monosaccharide pentoses and hexoses to trisaccharides using chemical derivatization and gas chromatography. If untreated large sugars are injected into the GC, they will degrade, and their alcohol groups would bind strongly or even permanently with the inner coating of certain chromatography columns. This would result in long retention times (>30 min) or a damaged column. Because this is both time-consuming and costly,

methods for protecting large and polar carbohydrates are currently in high demand. Analytical gas chromatographic studies serve as a vital technique for biological permeability studies and analysis of bodily fluids. Numerous methods for protecting mono- and oligosaccharides already exist and have validated the success of trimethylsilyl- (TMS) protecting groups[1,4,5].

Most effective protection methods commonly use silylate oximes, which are made from aldehyde or ketone[1,4]. Oxime formation is essential because certain reducing sugars can have open-, α, and β ring configurations (Figure 2). Multiple configurations of one type of molecule will cause diastereomers to be present. Diastereomers have different retention times on chromatograph and result in multiple peaks instead of one, thus decreasing GC sensitivity. This greatly complicates the quantification of that molecule and decreases its accuracy.



**β-D-lactulose**
Chemical Formula: $C_{12}H_{22}O_{11}$
Molecular Weight: 342.30

**Open-ring-D-lactulose**
Chemical Formula: $C_{12}H_{22}O_{11}$
Molecular Weight: 342.30

**α-D-lactulose**
Chemical Formula: $C_{12}H_{22}O_{11}$
Molecular Weight: 342.30

Figure 2: Three configurations of D-lactulose. A combination of open-ring and close rings (α and β) of lactulose can result in multiple peaks on the GC. Thus, it is best to keep the reducing sugars in an open ring.

Hydroxylamine hydrochloride is a reagent commonly used for oxime formation (Figure 3 and 4). The condensation reaction converts ketones and aldehydes into oximes, which locks reducing sugars, for example lactulose, in open-chain

configurations. This reaction yields an oxime and a water molecule. According to Le Chatelier's principle, the deprivation of a product (water) will shift the reaction toward the product side, increase yield, and thus enhance chromatographic signals.

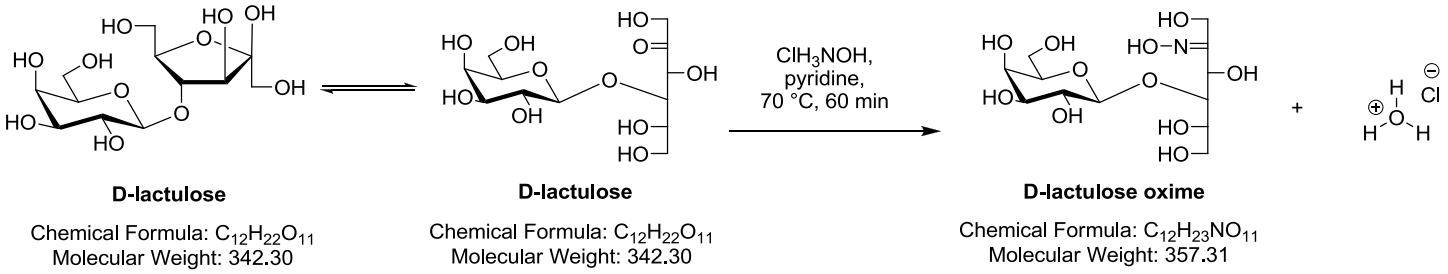



Figure 3: Example of oxime formation with hydroxylamine chloride. In this case, the starting carbohydrate is lactulose. As a reducing sugar, the fructose subunit of lactulose goes through close- and open-ring conformations. The oxime occupies the ketone on the fructose subunit and locks it open.

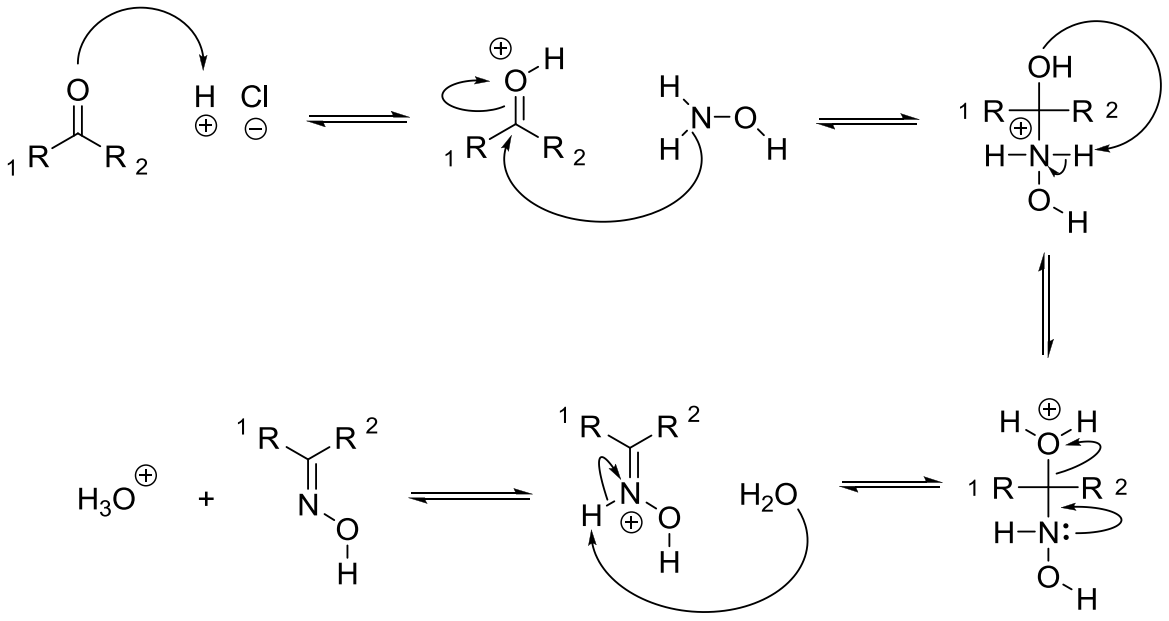


Figure 4: Mechanism of oxime formation. ($R_1$ can be an organic group, and $R_2$ represents an organic group or hydrogen.)

In previous studies, the oxime intermediate is allowed to react with 1-(trimethylsilyl)imidazole (Figure 5 and 6). The resulting trimethylsilyl groups on the lactulose derivative are excellent protecting groups because they are less reactive and are very non-polar compared to the alcohol groups on the initial carbohydrate. In addition, because they decrease the likelihood of hydrogen-bond formation, they increase the volatility of the product. An increase in volatility shortens the retention time and makes GC analysis less time-consuming.

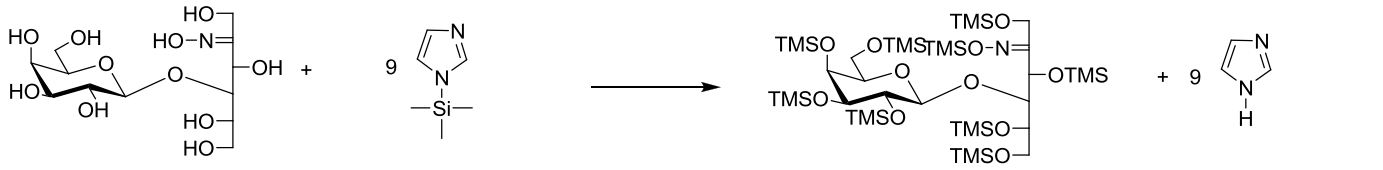



Figure 5: Example of silylation of a lactulose oxime with 1-(trimethylsilyl)imidazole (TMSI). When all the alcohol groups are protected with the bulky and non-polar TMS group, hydrogen bonds are less likely to form and the volatility of the product increases.

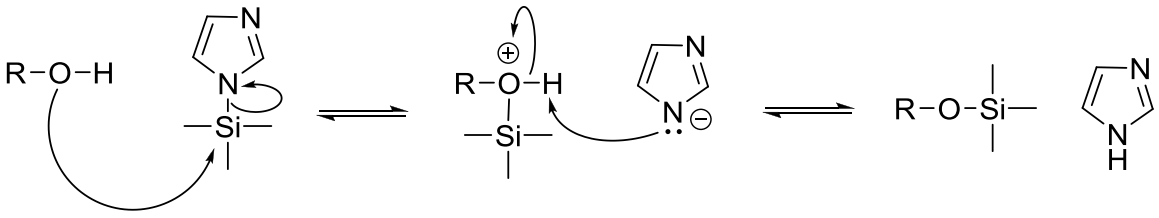
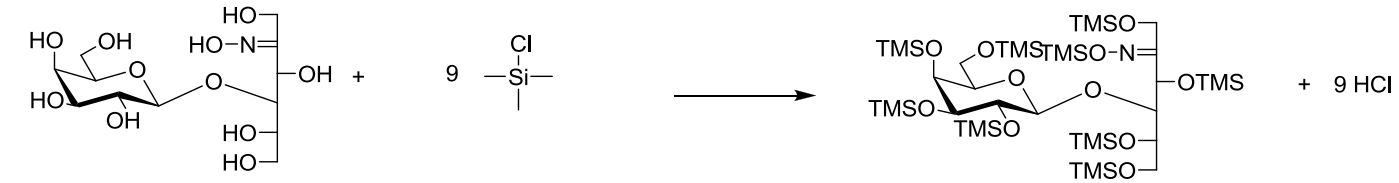


Figure 6: Mechanism of the silylation of an alcohol with TMSI. (R represents any organic group.)

Another silylating reagent, trimethylsilyl chloride (TMSCl) can be used in place of 1-(trimethylsilyl)imidazole (TMSI)[7] (Figure 7 and 8). The chloride ion within TMSCl is a much better leaving group in comparison to imidazole in (TMSI), which makes TMSCl more reactive. We intend to try this reagent in order to shorten the reaction time and, perhaps, increase the yield. However, this reagent does have limitations. First, a by-product of this reaction is hydrogen chloride gas, which forms hydrochloric acid upon entering the atmosphere. The acidic fumes are hard to control, and are corrosive to the human respiratory system and lab equipment. More importantly, the sugars can degrade under acidic reactions or conditions, creating background noises and diminished peak areas on the GC. While using TMSI is more time-consuming in comparison, we expect better results and cleaner reactions with less degradation.




**D-lactulose oxime**

Chemical Formula: $C_{12}H_{23}NO_{11}$
Molecular Weight: 357.31

**trimethylsilyl chloride**

Chemical Formula: $C_3H_9ClSi$
Molecular Weight: 108.64

Chemical Formula: $C_{39}H_{95}NO_{11}Si_9$
Molecular Weight: 1006.94

Figure 7: Example of silylation of a lactulose oxime with trimethylsilyl chloride (TMSCl).

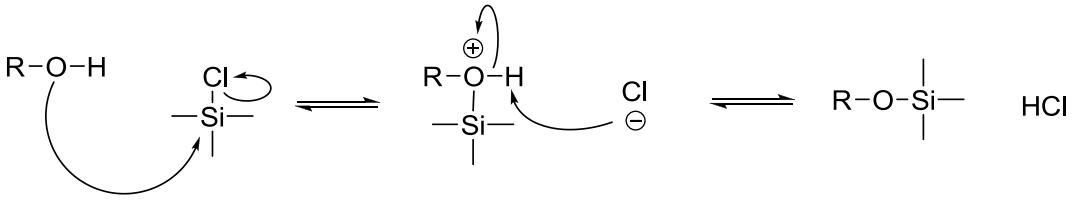



Figure 8: Mechanism of the silylation of an alcohol with TMSCl. (R represents any organic group.)

Pre-derivatization clean-up treatments are commonly performed by other researchers on samples of bodily fluids. These treatments include extractions with ethanol/water[3], methanol/water[7], or acetonitrile[3]. Post-derivatization clean-up treatments can also be applied. One article suggests evaporation followed by extraction with hexane[8]. The reagents used in extraction are especially vital to the biological studies done on a micro or nano scale. For accurate gas chromatographic analysis, the work-up procedure has to clean up unnecessary signals while leaving peak areas of interest undiminished. The polarity of the extraction reagents is essential in keeping this balance. After comparison and slight alterations, the post-derivatization treatment with concentration and extraction by hexane will be adapted. The non-polar nature of hexane makes it un-reactive and promises excellent solubility of the derivatized sugars.

Through trials of the all the reagents mentions above, we put together a derivatization procedure and applied it to three projects. The studies undertaken are the preparation of a synthetic mixture of 18 carbohydrates, analysis of carbohydrates contained in rat urine, and cellular permeability study of hyaluronic acid disaccharide (DiHA).

**Analysis of Synthetic Mixture of 18 Sugars**

This project aims to examine the separation capacity and the versatility of our derivatization procedure. We carefully selected 18 sugars following the example of Molnár-Perl and Horváth[6] (Figure 9). Within the carbohydrates used, there are alcohols, monosaccharides, disaccharides, trisaccharides, and a variety of their derivatives. These sugars include a variety of anomeric linkages, glycosidic bond types, and functional groups. This selection of carbohydrates is chosen to incorporate most characteristics of monosaccharides and oligosaccharides found in biological and non-biological systems. This diverse variety ensures that this method can be applied to other sugars and has the adaptability to work in any biological systems in future experiments.

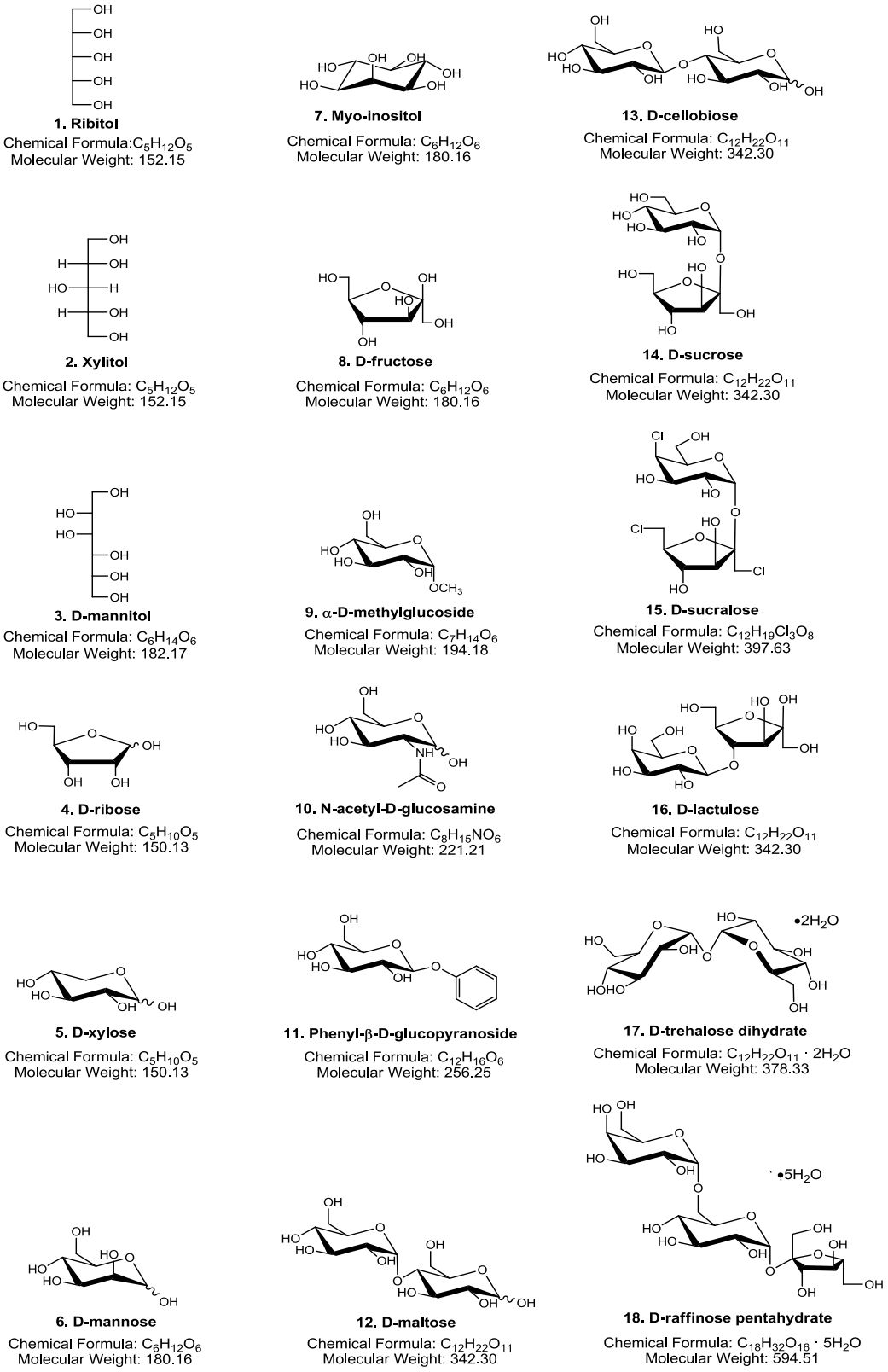



Figure 9: Carbohydrates for the synthetic 18-sugar analysis.

The basic derivatization scheme starts with the amination of a dried sample of all 18 sugars with hydroxylamine hydrochloride diluted in pyridine. Gentle heating with slight ventilation allows reducing sugars to transform into oxime and water to evaporate, thus driving the equilibrium toward the oxime formation while keeping sugars in solution. The next step is the silyl ether formation on the oxime and alcohols with 1-(trimethylsilyl)imidazole. The solution was again allowed to react while heated. After the subsequent evaporation, the supernatant was extracted with hexane and saturated hydroxylamine solution. The saturated hydroxylamine solution can trap polar impurities, and the top hexane layer can accommodate non-polar sugar derivatives.

In a chromatograph, each of the 18 sugars' presence will be identified by their retention time (Table 1).

| No. | Sugar | Retention Time (min) |
|---|---|---|
| 1 | Ribitol-TMS | 9.694-9.739 |
| 2 | Xylitol-TMS | 9.444-9.619 |
| 3 | Mannitol-TMS | 10.763-11.021 |
| 4 | Ribose-oxime-TMS | 10.050-10.099 |
| 5 | Xylose-oxime-TMS | 9.886-9.927 |
| 6 | Mannose-oxime-TMS | 11.301-11.348 and 11.422-11.498* |
| 7 | Myo-inositol-TMS | 11.864-11.920 |
| 8 | Fructose-oxime-TMS | 11.034-11.072 and 11.083-11.127* |
| 9 | α-D-methylglucoside-TMS | 10.643-10.683 |
| 10 | N-acetyl-D-glucosamine-oxime-TMS | 12.005-12.452** |
| 11 | Phenyl-β-glucopyranoside-TMS | 12.948-12.985 |
| 12 | Maltose-oxime-TMS | 16.005-16.554** |
| 13 | Cellobiose-oxime-TMS | 15.788-15.893 |
| 14 | Sucrose-TMS | 14.705-14.777 |
| 15 | Sucralose-TMS | 15.148-15.219 |
| 16 | Lactulose-oxime-TMS | 15.653-15.755 |
| 17 | Trehalose dihydrate-TMS | 15.405-15.494 |
| 18 | Raffinose pentahydrate-TMS | 17.224-17.569 |

Table 1: Retention times used to identify each carbohydrate within the gas chromatograph. Some sugars appear as two distinct peaks* or multiple peaks** due to oxime geometry.

The chromatograph for this particular project has to show the clear and separated peak of each sugar (Figure 10). An informal serial dilution was also done with this 18-sugar solution to develop a resolute chromatograph. The resolution of a sample can be affected by an overlap of peaks in concentrate samples or by low signals (abundance <100). An overlap of signals will interfere with accuracy and quantification.

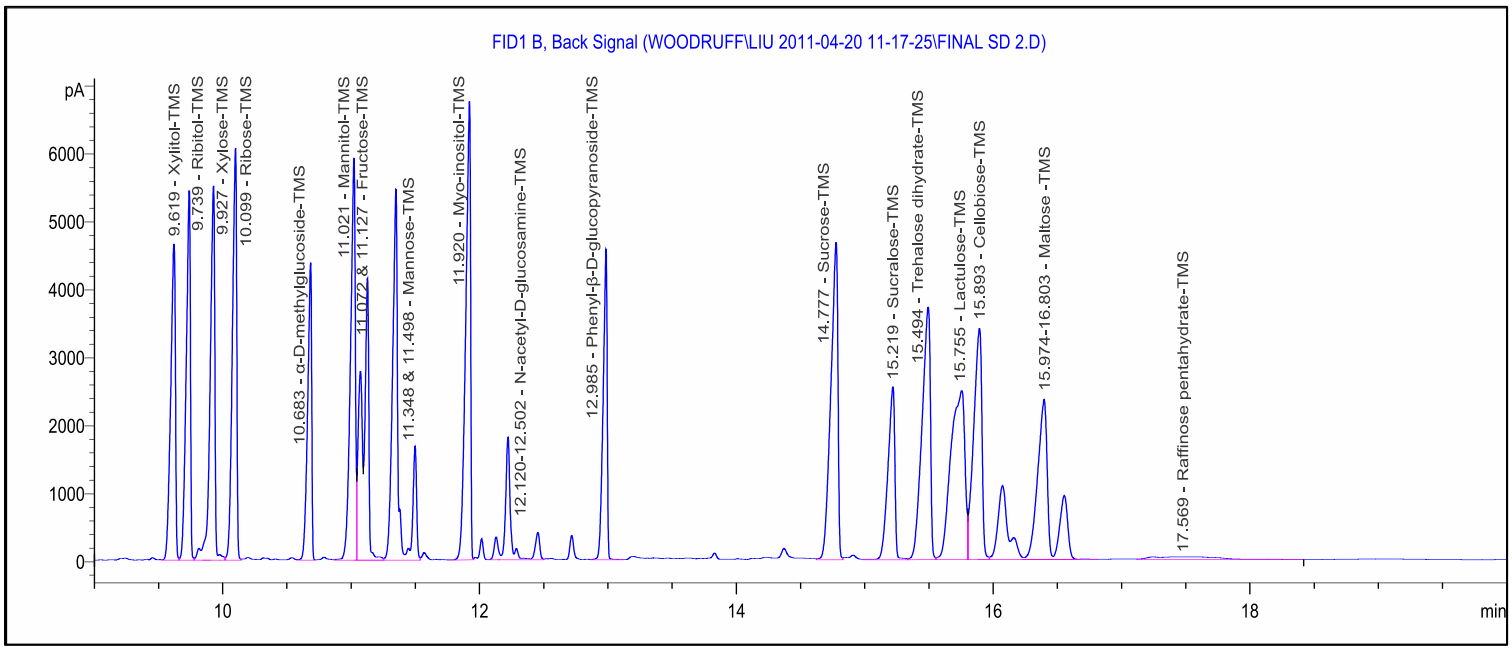



Figure 10: Typical chromatograph of equal concentrations (50 mg/L) of all 18 sugars.

This project has shown that this derivatization scheme can be applied to a large number of different compounds. The separation will allow for quantitative testing of large quantities of compounds, making it more accurate and less time-consuming.

**Rat Urine Analysis**

Our method was initially designed for this project in order to qualify and quantify certain sugar content within rat urine. The derivatization procedure was put together by attempting and modifying several methods from previously-published papers related to intestinal permeability. Among all three projects, this is the earliest and most detailed study we have performed with our method. The sugars involved in this study are very important because they are the most popularly used marker for permeability studies as described in the introduction. Having the capacity to analyze these sugars accurately can make this method applicable to more biological systems for understanding metabolism and permeability. However, current methods have unimpressive signal-to-noise ratios and major problems with reproducibility. For example, purification methods being applied to urine, such as ethanol/water or acetonitrile[3] extraction, display low signal-to-noise and mediocre resolution of sugar probes at best (Figure 11 and 12).

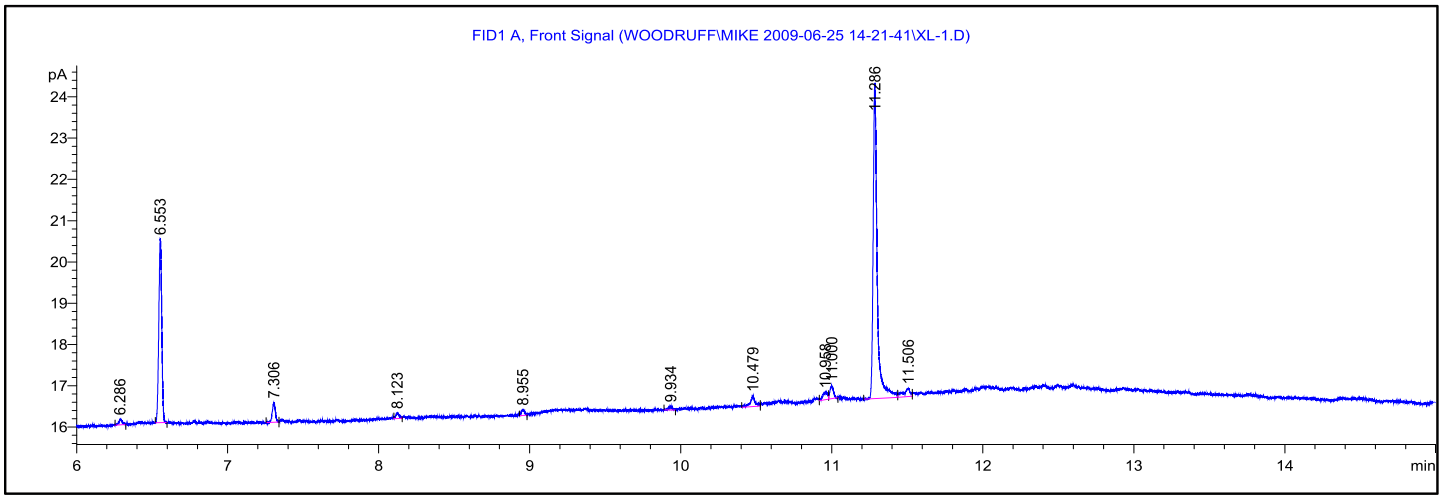



Figure 11: Rat urine cleaned with ethanol/water solution. We are not able to identify any peaks due to low abundance signal.

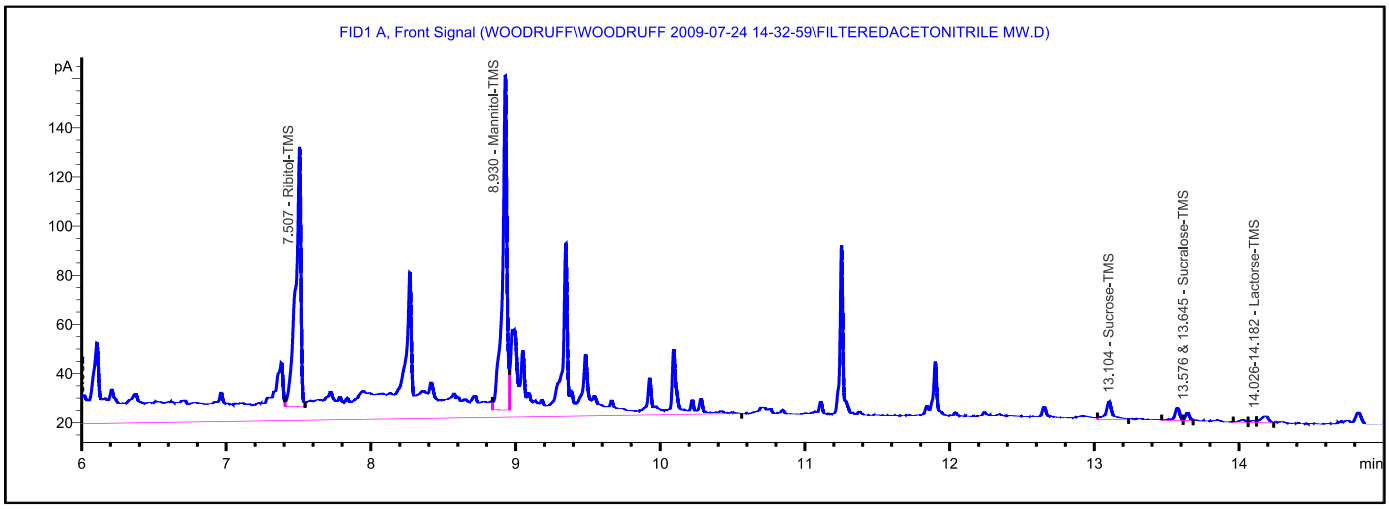



Figure 12: Rat urine cleaned with acetonitrile. This is our most resolute acetonitrile trial. The abundance signal is still relatively low in comparison to the chromatograph of urine cleaned with a hexane extraction.

Non-endogenous sugars, such as sucrose, mannitol, lactulose, and the recently discovered sucralose are preferred probes for animal model or human permeability studies[1] (Figure 13). To examine gastrointestinal permeability, these non-metabolizable sugars can be ingested and absorbed at various rates transcellularly and/or paracellularly in the small and/or large intestines. The absorbed fraction then enters the blood stream and is subsequently excreted in urine. The amount of each sugar recovered from healthy rat urine was found experimentally to be 82.5 ± 8.4% for sucrose, 97.3 ± 10.9% for mannitol, 72.1 ± 3.7% for lactulose, and 76.8 ± 6.7% for sucralose[3]. For rats with intestinal epithelial or glomerular damage, these values are likely to increase. Dr. Zhou from The Ohio State University utilized these markers to study intestinal permeability in force-fed diabetic rats, *Rattus norvegicus*[7], with gastrointestinal disorders. The four sugars mentioned above are subjected to our derivatization and GC analysis

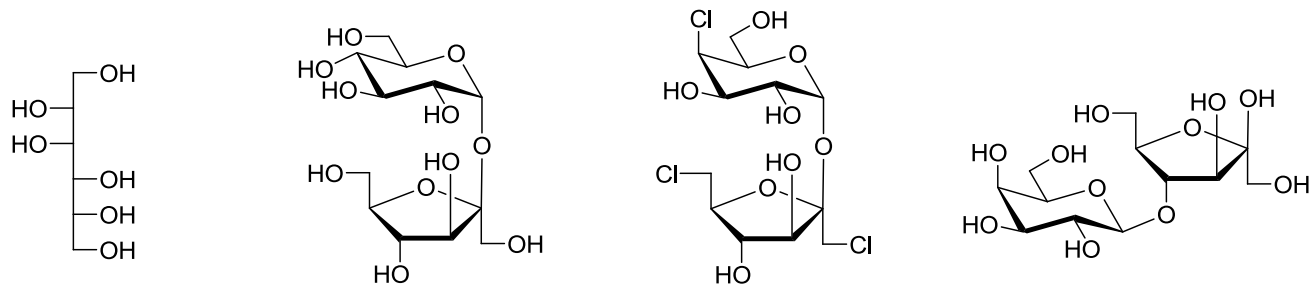



Figure 13: Structures of D-mannitol, D-sucrose, D-sucralose, and D-lactulose.

The retention time of each sugar including the internal standard, ribitol, which will be used for quantification, is detected individually. The retention time serve as a tool to identify each peak on a gas chromatograph.

| Sugar | Retention Time (min) |
|---|---|
| Ribitol-TMS | 7.403-7.527 |
| Mannitol-TMS | 8.807-8.954 |
| Sucrose-TMS | 12.942-13.135 |
| Sucralose-TMS | 13.394-13.606 |
| Lactulose-oxime-TMS | 13.895-14.187* |

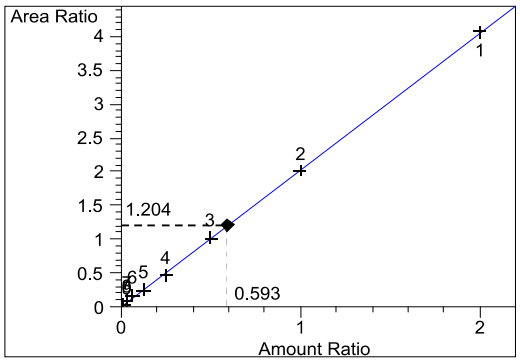
Table 2: List of the retention times of the derivatized sugars. *The two isomers of lactose oxime cause the appearance of two wide peaks.

The quantification of these carbohydrates in urine is performed using nine 2-fold serial dilutions. They are spiked to the same concentration of an internal standard (ribitol, 100 mg/L) to make various ratios, which is the concentration of a sugar of interest divided by that of the internal standard. These ratios are used to construct the x-axis of calibration curves.

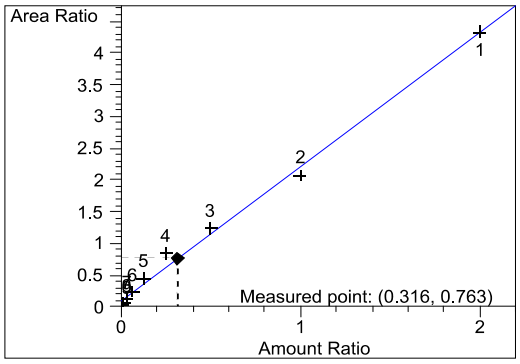
| Serial Dilution No. | Ribitol Internal Standard (mg/L) | Mannitol, Sucrose, Sucralose, and Lactulose (mg/L) | Amount ratio |
|---|---|---|---|
| 1 | 100 | 196.08 | 1.96 |
| 2 | 100 | 98.04 | 0.980 |
| 3 | 100 | 49.02 | 0.490 |
| 4 | 100 | 24.51 | 0.245 |
| 5 | 100 | 12.25 | 0.123 |
| 6 | 100 | 6.13 | 0.0613 |
| 7 | 100 | 3.06 | 0.0306 |
| 8 | 100 | 1.53 | 0.0153 |
| 9 | 100 | 0.77 | 0.0077 |

Table 3: Standard concentrations within serial dilutions. Dilutions are numbered 1-9. The amount ratio is of a sugar of interest and ribitol. This ratio allows for quantification.

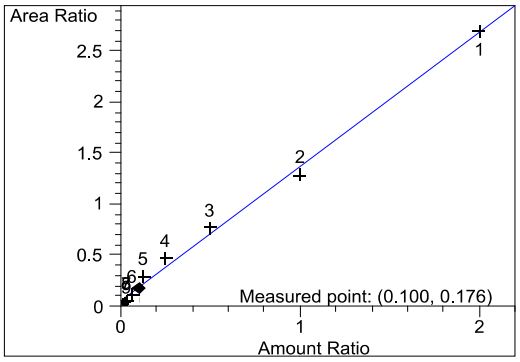
A typical calibration curve is made from serial dilutions and their chromatographs. It consists of the amount ratio for its *x-axis* and area ratio for its *y axis.* Whereas the data for the amount ratio is known when the serial dilution is made, the data for the area ratio is collected from the resulting chromatographs. An area ratio comes from dividing the area under each peak of interest, which are mannitol, sucrose, sucralose, and lactulose, by the area under the ribitol internal standard. The area and amount ratio has a linear relationship and can be described by the equation: $y = mx + b$. All calibration curves have correlation values greater than 0.99, which indicates that there are minimal deviations to the value and that the method can derive a wide range of concentrations (0.77 -196 mg/L) consistently.



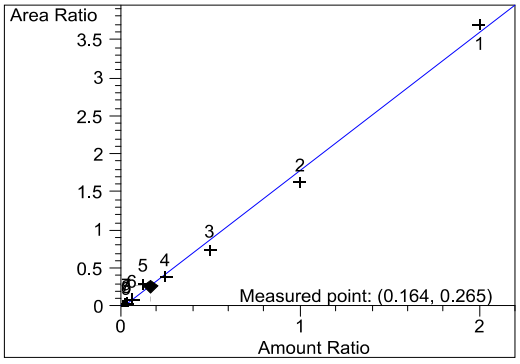

Mannitol-TMS at exp. RT: 8.807
FID2 B, Back Signal
Correlation: 0.99989
Residual Std. Dev.: 0.02085
Formula: y = mx + b
m: 2.02723
b: 8.47866e-4
x: Amount
y: Area




Sucrose-TMS at exp. RT: 12.918
FID2 B, Back Signal
Correlation: 0.99684
Residual Std. Dev.: 0.11509
Formula: y = mx + b
m: 2.11227
b: 9.44541e-2
x: Amount
y: Area




Sucralose-TMS at exp. RT: 13.393
FID2 B, Back Signal
Correlation: 0.99771
Residual Std. Dev.: 0.06131
Formula: y = mx + b
m: 1.32180
b: 4.34641e-2
x: Amount
y: Area



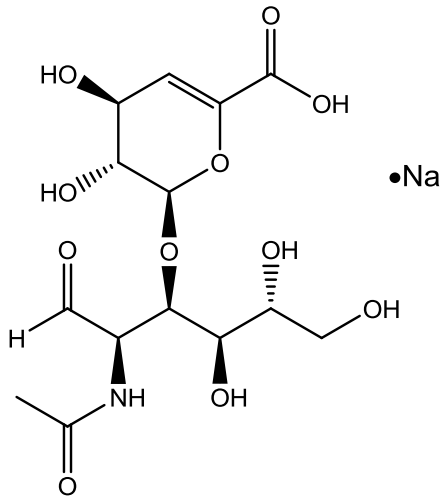

Lactulose-TMS at exp. RT: 14.002
FID2 B, Back Signal
Correlation: 0.99731
Residual Std. Dev.: 0.09096
Formula: y = mx + b
m: 1.81154
b: -3.25504e-2
x: Amount
y: Area

Figure: 11 Calibration curves for the carbohydrates of interests. The high correlation value shows that the derivatization procedure can be applied across a wide range of concentrations (from 196 mg/L to 0.77 mg/L).

From these plots, we can determine the theoretical value of that particular gas chromatographic system, in which the chromatographs are developed, by finding the x-intercepts. Also, we can quantify actual urine samples by looking at their own area ratios and extrapolating the corresponding amount ratio. Because the concentration of ribitol stays constant, we can simply multiply the resultant amount ratio with 100 mg/L of ribitol to arrive at the actual concentration of a sugar of interest within a sample of urine.

**Cellular Permeability of Hyaluronic Acid Disaccharide**

The disease-causing *Streptococcus pneumoniae* requires carbohydrates to survive, grow, and colonize the airway of the human body. Even though free monosaccharides are scarce in the airway, N-linked glycans, O-linked glycans, and glucosaminoglycans are abundant. *Streptococcus pneumoniae* is able to use glucosaminoglycans, namely hyaluronic acid (HA), as an important energy source[10]. Hyaluronic acid has repeating disaccharide units, which comprise glucuronic acid and N-acetylglucosamine. To transport such a massive and polymerized molecule into the cell, *Streptococcus pneumoniae* is able to cleave HA into hyaluronic acid dissacharides (DiHA) with hyaluronate lyase (Figure 12). We are studying the cellular permeability of this dissacharide.




**Hyaluronic Acid Disaccharide**
Chemical Formula: $C_{14}H_{20}NO_{11}Na$
Molecular Weight: 401.30

Figure 12: Structure of hyaluronic acid disaccharide.

*Streptococcus pneumoniae* are treated at Nationwide Children's Hospital. After a timed exposure to hyaluronic acid disaccharides, the cells are lysed. Other than

controls, the samples included supernatants and lysed cellular samples (wild-type or mutant *Streptococcus pneumoniae*) that vary in time of exposure to hyaluronic acid disaccharide.

| Sample Description | DiHA Content | Sample Description | DiHA content |
|---|---|---|---|
| WT, 0 min, supernatant<br>WT, 15 min, supernatant<br>WT, 30 min, supernatant<br>WT, 60 min, supernatant | Relative decrease in concentration down the column | WT, 0 min, cells<br>WT, 15 min, cells<br>WT, 30 min, cells<br>WT, 60 min, cells | Relative increase in concentration down the column |
| No cells, media control | No DiHA | HA control | DiHA present |

Table 4: This is a table given by the Children's Hospital that described a batch of samples derived from a wild type of *Streptococcus pneumoniae*. This set of samples compares content of hyluronic acid disaccharide of the supernatant with that of the lysed cells. The permeability of the wild type *Streptococcus pneumoniae* will be compared to that of mutant strains in future experiments.

Again, the samples are derivatized with an internal standard (ribitol, 100 mg/L) and analyzed by GC and GC/MS. For the Children's Hospital, the time of exposure to hyaluronic acid would be their variable. The quantification of this project is still under study.

## Results and Discussion

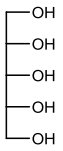
Through exploring multiple eligible chemicals, reagents found suitable to derivatize mono-, di-, and trisaccharides comprised 10% solution of hydroxylamine in anhydrous pyridine for oxime formation, 1-(trimethylsilyl)imidazole for silylation, and hexane coupled with a saturated hydroxylamine solution for extraction.

While the separation of the synthetic mixture of 18 sugars and the results of the rat urine analysis are conclusive, the quantification of the synthetic mixture of 18 sugars is in progress. The cellular permeability project of hyaluronic acid disaccharide is still continuing as we are awaiting a new set of cellular samples for derivatization and GC testing.

It is important to note that the retention times for some carbohydrates are reported as a range. This is because their values are subjected to slight shifts when solutions vary in concentration. This is especially true for more concentrated serial dilutions to have a higher retention time. Lastly, there are two GC types (GC/FID and GC/MS) used in these projects. Their retention times for the same molecule may be dramatically different, but the order of separation and area ratios for quantifications are comparable.

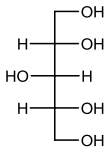
**Analysis of Synthetic Mixture of 18 Sugars**

This project is designed to test the versatility of this derivatization procedure with a wide variety of carbohydrates. These sugars are uniformly derived by the same procedure and quantity of reagents (Figure 13).



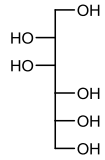
**1. Ribitol**

Chemical Formula:$C_5H_{12}O_5$
Molecular Weight: 152.15



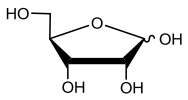
**2. Xylitol**

Chemical Formula: $C_5H_{12}O_5$
Molecular Weight: 152.15



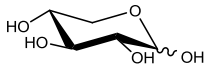
**3. D-mannitol**

Chemical Formula: $C_6H_{14}O_6$
Molecular Weight: 182.17



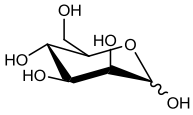
**4. D-ribose**

Chemical Formula: $C_5H_{10}O_5$
Molecular Weight: 150.13



**5. D-xylose**

Chemical Formula: $C_5H_{10}O_5$
Molecular Weight: 150.13

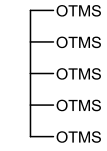


**6. D-mannose**

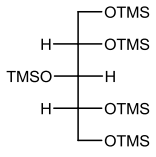
Chemical Formula: $C_6H_{12}O_6$
Molecular Weight: 180.16

1. Evaporate to Dryness
2. $ClH_3NOH$, pyridine, 70 °C, 1 hr
3. TMS-Imidazole, 70 °C, 30 min
4. Evaporate
5. 150 µL Hexanes, 100 µL Saturated Pyridine
6. Extract 50 µLof Hexane Layer
7. 1µL Injection into GC/MS

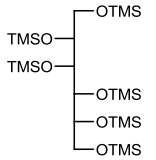




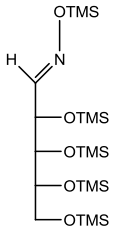
Chemical Formula: $C_{20}H_{52}O_5Si_5$
Molecular Weight: 513.05



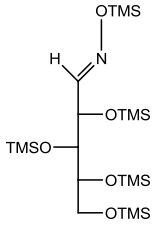
Chemical Formula: $C_{20}H_{52}O_5Si_5$
Molecular Weight: 513.05



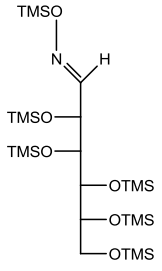
Chemical Formula: $C_{24}H_{62}O_6Si_6$
Molecular Weight: 615.26



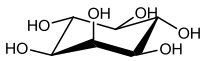
Chemical Formula: $C_{20}H_{51}NO_5Si_5$
Molecular Weight: 526.05



Chemical Formula: $C_{20}H_{51}NO_5Si_5$
Molecular Weight: 526.05

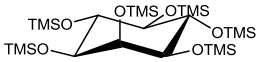


Chemical Formula: $C_{24}H_{61}NO_6Si_6$
Molecular Weight: 628.26

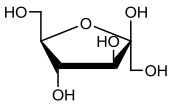


**7. Myo-inositol**

Chemical Formula: $C_6H_{12}O_6$
Molecular Weight: 180.16

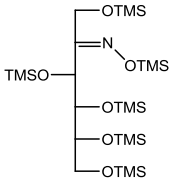


Chemical Formula: $C_{24}H_{60}O_6Si_6$
Molecular Weight: 613.24

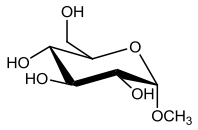


**8. D-fructose**

Chemical Formula: $C_6H_{12}O_6$
Molecular Weight: 180.16

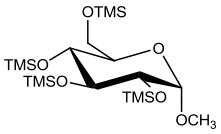


Chemical Formula: $C_{24}H_{61}NO_6Si_6$
Molecular Weight: 628.26



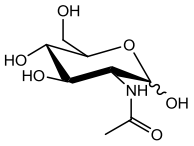
**9. α-D-methylglucoside**

Chemical Formula: $C_7H_{14}O_6$
Molecular Weight: 194.18



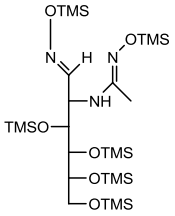
Chemical Formula: $C_{19}H_{46}O_6Si_4$
Molecular Weight: 482.91

1. Evaporate to Dryness
2. $ClH_3NOH$, pyridine, 70 °C, 1 hr
3. TMS-Imidazole, 70 °C, 30 min
4. Evaporate
5. 150 µL Hexanes, 100 µL Saturated Pyridine
6. Extract 50 µLof Hexane Layer
7. 1µL Injection into GC/MS

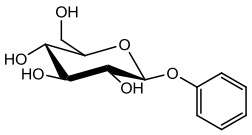


**10. N-acetyl-D-glucosamine**

Chemical Formula: $C_8H_{15}NO_6$
Molecular Weight: 221.21

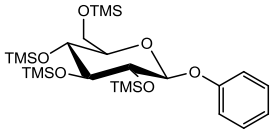


Chemical Formula: $C_{26}H_{65}N_3O_6Si_6$
Molecular Weight: 684.32

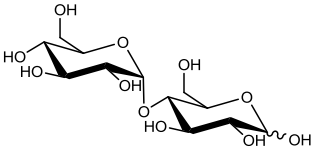


**11. Phenyl-β-D-glucopyranoside**

Chemical Formula: $C_{12}H_{16}O_6$
Molecular Weight: 256.25

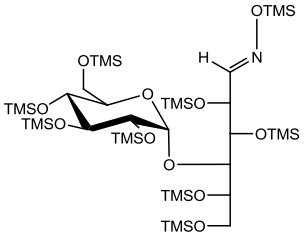


Chemical Formula: $C_{24}H_{48}O_6Si_4$
Molecular Weight: 544.98



**12. D-maltose**

Chemical Formula: $C_{12}H_{22}O_{11}$
Molecular Weight: 342.30



Chemical Formula: $C_{39}H_{95}NO_{11}Si_9$
Molecular Weight: 1006.94

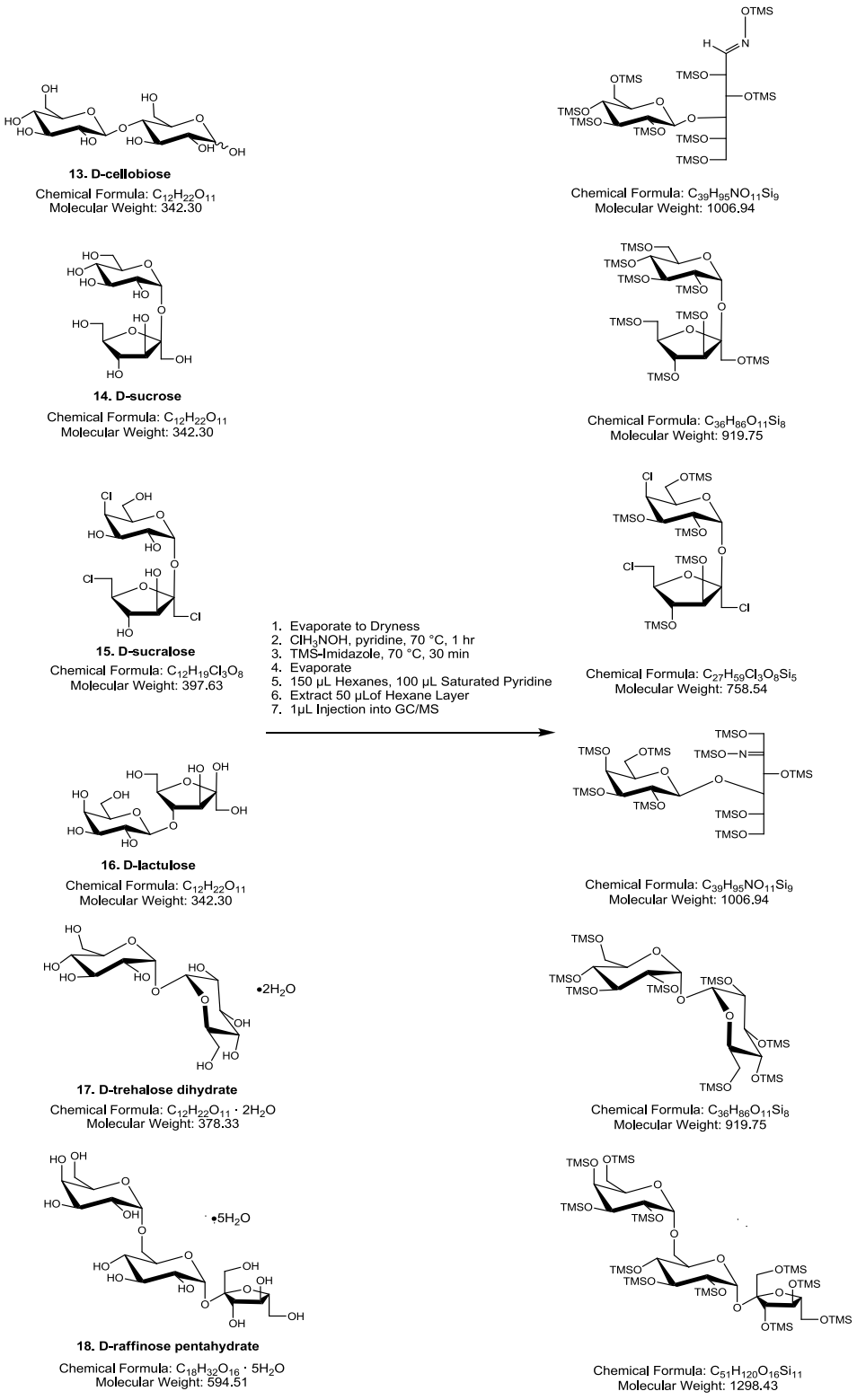



Figure 13: This is a derivatization scheme for all 18 sugars.

To identify these molecules, each sugar (100 mg/L) was derived and analyzed by GC-FID system separately. Combined with the data from nine 2-fold serial dilutions of mixtures of all 18-carbohydrates, we determine the range of retention times based on concentrations. For concentrations between 0.78 mg/L and 200 mg/L, their retention times are: **1**. ribitol (9.694-9.739 min), **2**. xylitol (9.444-9.619 min), **3**. D-mannitol (10.763-11.021 min), **4**. D-ribose (10.050-10.099 min), **5**. D-xylose (9.886-9.927 min), **6**. D-mannose (first peak: 11.301-11.348 min, second peak: 11.422-11.498 min), **7**. myo-inositol (11.864-11.920 min), **8**. D-fructose (first peak: 11.034-11.072 min, second peak: 11.083-11.127 min), **9**. α-D-methylglucoside (10.643-10.683 min), **10**. N-acetyl-D-glucosamine (multiple peaks: 12.005-12.452 min), **11**.Phenyl-β-glucopyranoside (12.948-12.985 min), **12**. D-maltose (multiple peaks: 16.005-16.554), **13**. D-cellobiose (15.788-15.893 min), **14**. D-sucrose (14.705-14.777 min), **15**. D-sucralose (15.148-15.219 min), **16**. D-lactulose (15.653-15.755 min), **17**. D-trehalose dihydrate (15.405-15.494 min), and **18**. D-raffinose pentahydrate (wide peak around 17.224-17.569 min).

The purpose of the serial dilution mentioned previously was to develop a resolute chromatograph, in which the concentration neither causes overwhelming overlaps of peaks nor low signal intensity. This study consists of a wide variety of sugars ranging from monosaccharide to trisaccharide. Because of the large number and diversity of molecules that participate in one sample, we have observed that the concentration range between displaying excessive signal overlaps or undependable low signals is narrow (between approximately 12.5-100 mg/L). Even though the samples' theoretical detection limit may be extremely low (predictably between 0 ng/mL and 25 ng/mL),

dilutions with concentrations between 12.5 mg/L and 100 mg/L coupled with a resolute internal standard would be ideal for constructing a calibration curve.

**Rat Urine Analysis**

This project is used to develop the derivation method. A pure sample of each sugar including the intended internal standard is run to determine the retention time for composition of the urine samples. A synthetic combination of all 5 carbohydrates was also run to show an overall pattern that would be expected (Figure 14).

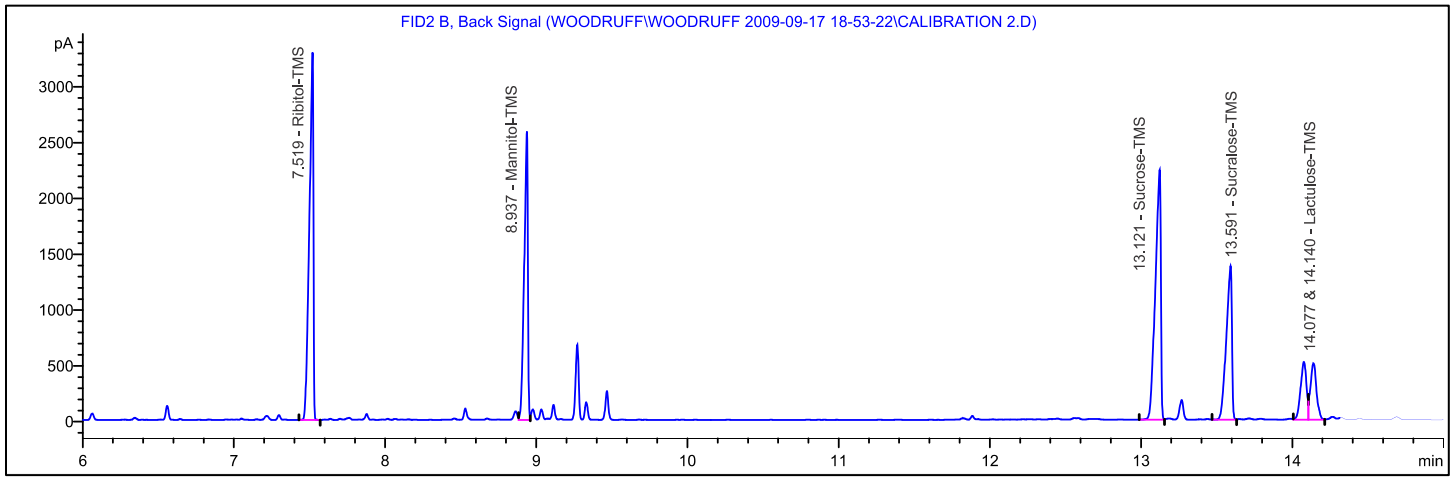



Figure 14: Five carbohydrates in a synthetic mixture (100 mg/L per sugar).

Ribitol, the internal standard, has a retention time between 7.403 and 7.527 min. The sugars of interest are mannitol, sucrose, sucralose, and lactulose. Their retention times are around 8.807-8.954 min, 12.942-13.135 min, 13.394-13.606 min, and 13.895-14.187 min respectively.

To quantify each sugar, nine 2-fold serial dilutions are made from a synthetic combination of all 5 carbohydrates. The concentration of sugars of interest ranges from 196 mg/L per sugar to as low as 0.77 mg/L, while the concentration of the ribitol internal

standard stays the same (100 mg/L). The chromatographs of the serial dilution are used to construct calibration curves (Figure 11). A high correlation value (>0.99) shows that all plots used to construct the calibration curves have minimal deviations. This value supports the consistency of detection across a multitude of concentrations. Not only does this comment on the accuracy of the equipment, but also the precision and versatility of the derivatization procedure.

The theoretical detection limits of the sugars of interest are determined by multiplying the x-intercept values with the 100 mg/L concentration of the internal standard. Their values are: mannitol: 0.0418 ng/mL, sucrose: 4.4717 ng/mL, sucralose: 3.2883 ng/mL, and lactulose: 1.7968 ng/mL.

The actual urine samples are spiked to 100 mg/L of internal standard, derivatized, and analyzed by flame GC (Figure 15).

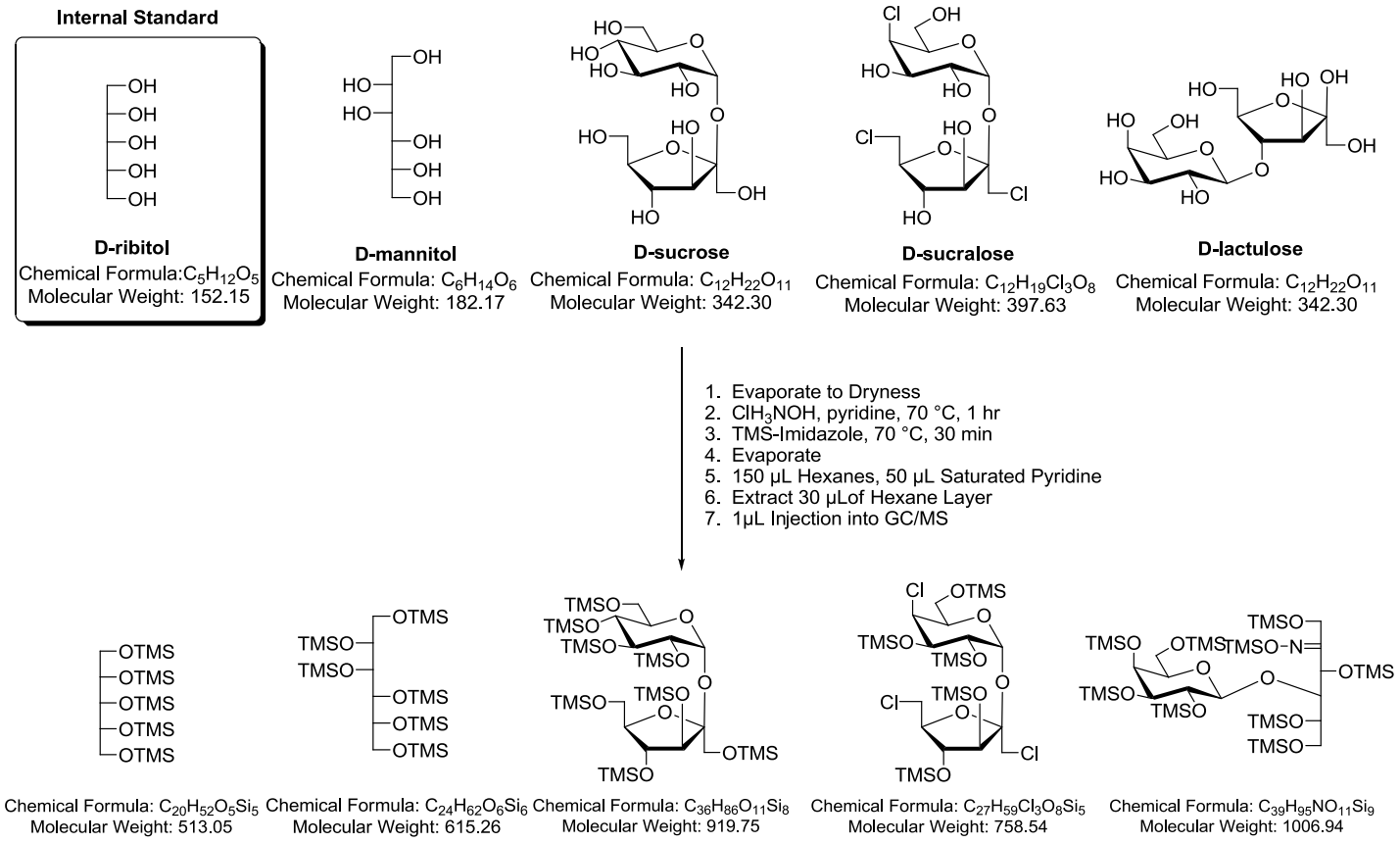



Figure 15: Derivatization scheme of mannitol, sucrose, sucralose, lactulose, and the internal standard, ribitol.

On a first glace at the chomatographs, the quality of the peaks can be assessed roughly (Figure 16). Due to the oxime formation, which keeps reducing sugar, lactulose, in the open-ring conformation, the signal is more resolute. Multiple or shouldered peaks of lactulose are merely a result of diastereoisomers, which cannot be altered, and the wide peaks must be added to calculate the sugar's total area (Figure 17). Another apparent aspect worth noting is the effectiveness of this method at cleaning up biological materials. Because most renal excretions are polar, the non-polar hexane successfully isolates the few non-polar metabolites along with all of the non-polar sugar derivatives.

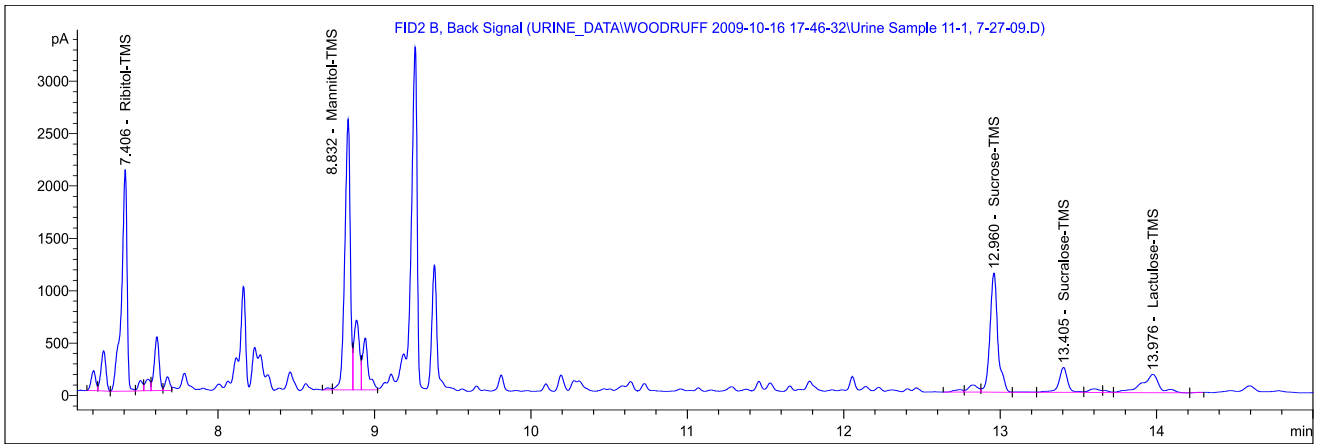



Figure 16: A typical urine analysis for sugar identification and concentration. Notice how this procedure is able to gently clean up the background noise while not diminishing the signals of interest.

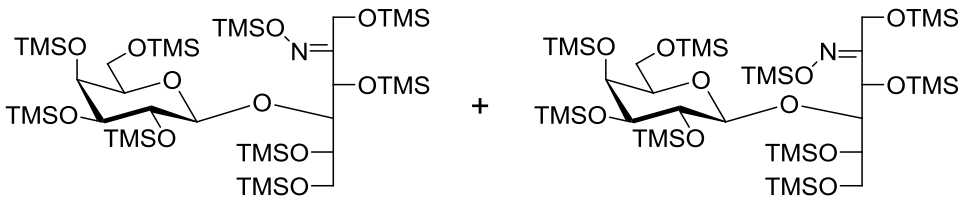



Figure 17: E and Z diastereoisomers of the silylated oximes of lactulose.

Hard data from the urine samples are also put in an area ratio – (sugar of interest) / (internal standard). Using this ratio and the already-constructed calibration curve, the amount ratio of each of the sugar of interest is extrapolated. The approximate concentration of each sugar can be derived by multiplying the amount ratio with 100 mg/L of internal standard, ribitol.

Due to the sheer quantity of the urine samples, only a proportion of the samples are shown in this thesis. However, this should be sufficient in demonstrating the efficiency of this derivatization procedure at qualifying and quantifying complex bodily excretions. The calibration curve and the quantification from hundreds of urine samples are submitted and validated by external statistical analysis at The Ohio State Medical Center (Table 5).

| Sample Number | Sample Type | Mannitol (ng/mL) | Sucrose (ng/mL) | Sucralose (ng/mL) | Lactulose (ng/mL) | [Lactulose]/ [Mannitol] |
|---|---|---|---|---|---|---|
| 25-1, 7-32-09 | Control | 44.2 | 6.9 | 18 | 12.1 | 0.27 |
| 30-1, 7-14-09 | Control | 39.6 | 13.6 | 0.8 | 10.9 | 0.28 |
| 24-1, 7-31-09 | Control | 48.5 | 21.3 | 3 | 9.9 | 0.20 |
| 1-1, 6-25-09 | Type 1 | 65.2 | 21.8 | 18.7 | 51.9 | 0.80 |
| 2-1, 6-25-09 | Type 1 | 50.3 | 22.8 | 9.9 | 37.6 | 0.75 |
| 3-1, 6-26-09 | Type 2 | 48.2 | 10.2 | 10.1 | 40.5 | 0.84 |
| 4-1, 6-26-09 | Type 2 | 71.3 | 43.4 | 19.8 | 49.4 | 0.69 |
| 4-3, 7-8-09 | Type 3 | 64.8 | 111 | 7 | 59.2 | 0.91 |
| 5-1, 6-26-09 | Type 3 | 46.5 | 22.5 | 9.6 | 53 | 1.14 |

Table 5: Quantification of 4 sugars of interest. The ratio of the concentration of lactulose over that of mannitol reflects the degree of intestinal permeability. In healthy control rats, this ratio ranges between 0.20 and 0.40. In rats with gastrointestinal disorders (type 1-3), this ratio increases, which indicates various degree of increase in intestinal permeability. This table merely displays a set of hundreds of samples we analyzed.

**Cellular Permeability of Hyaluronic Acid Disaccharide**

We are assisting Nationwide Children's Hospital in Columbus in finding the permeability of hyaluronic acid disaccharide (DiHA) in living *Streptococcus pneumoniae*. The retention time of a pure hyaluronic acid derivative (100 mg/L and 20 mg/L) is between 15.7 and 17.5 min on GC/MS system (6850/5975C) and between 19.4 and 20.2 min on flame GC system (7890A). Because this molecule is a disaccharide, the apparent wide peak at a large retention time is not unexpected.

The cells are treated in Nationwide Children's Hospital. After a timed exposure to hyaluronic acid (0, 15, 30, and 60 min), the cells are lysed. The samples come in three catagories: supernatant of either wild type or mutant *Streptococcus pneumoniae*, lysed *Streptococcus pneumoniae* solutions, and controls (a media solution of DiHA and a cellular sample without DiHA).

The samples are spiked to 100 mg/L ribitol internal standard, derived, and tested by GC and GC/MS (Figure 18).

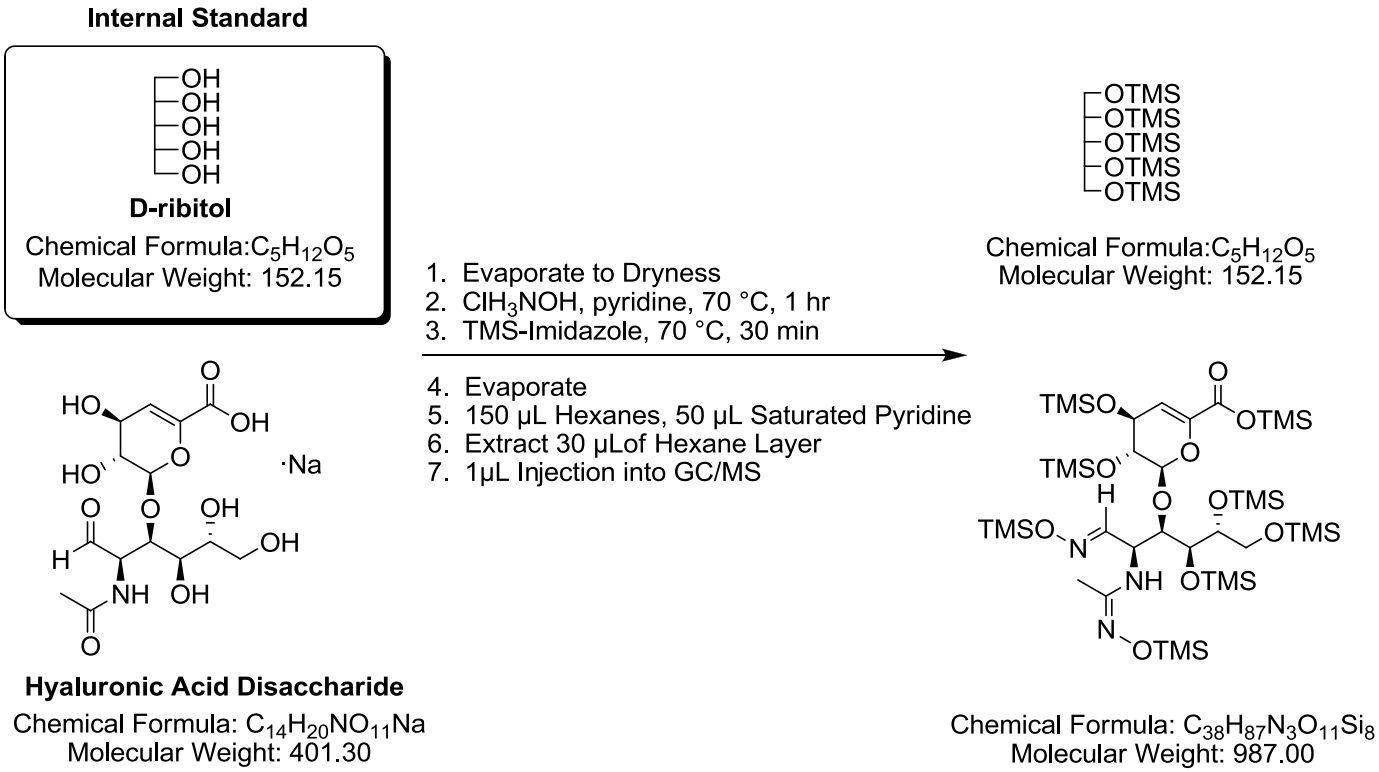



Figure 18: Derivatization of hyaluronic acid disaccharide.

The peaks for hyaluronic acid from both the control and the supernatant samples resolve. Due to extremely low concentrations of hyaluronic acid disaccharide within the lysed cells, the lysed cellular solutions tend to show less resolute hyaluronic acid disaccharide peaks (Figure 19). However, this should not be a problem for analysis of cellular permeability as long as the total hyaluronic acid disaccharides added and its concentration within the supernatants of the corresponding time of exposure are known (Figure 20).

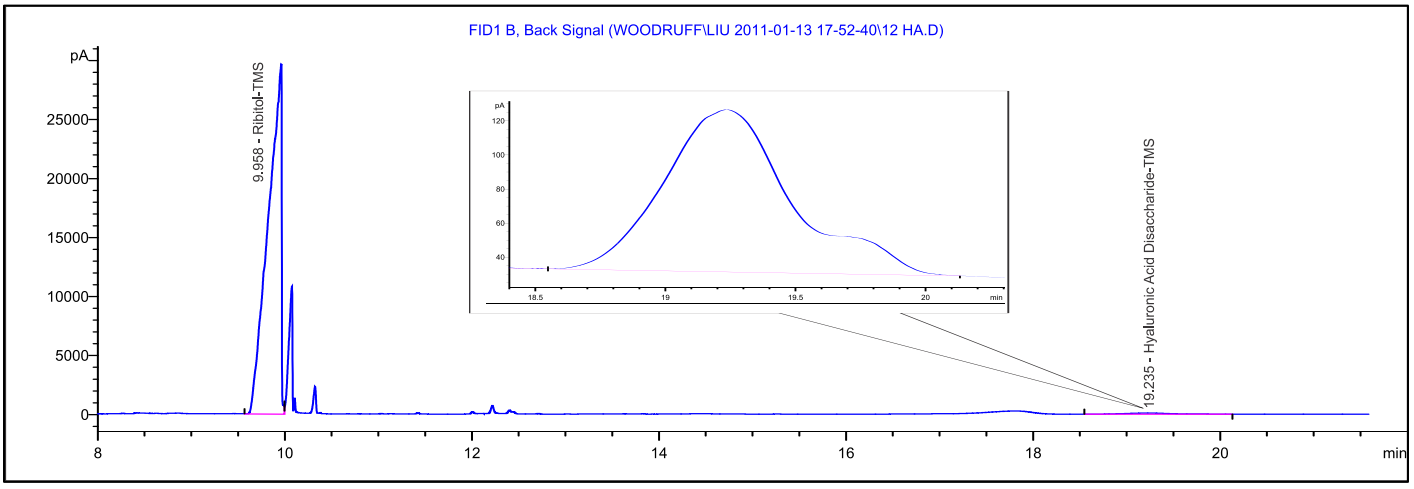



Figure 19: A chromatograph of lysed cells at 60 minutes ran on the GC. Notice the small Hyaluronic acid disaccharide peak at 19.235 min.

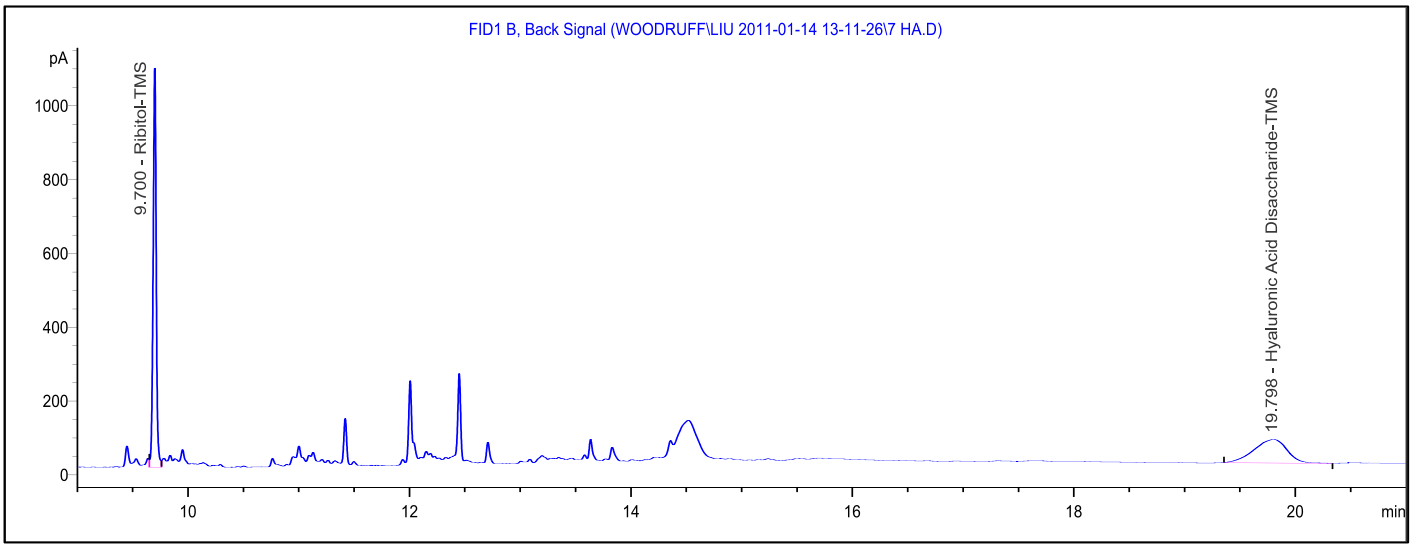



Figure 20: A chromatograph of supernatant at 60 minutes ran on the GC. The small amount absorbed by the cell can be derived from the concentration of hyaluronic acid in the supernatant.

In the control samples, hyaluronic acid and the internal standard, ribitol, are clearly illustrated (Figure 21). However, in cellular media or supernatants, the result can be clouded by cellular membrane components. Because their fatty acid chains are non-polar, these molecules tend to dissolve well in the hexane layer during extraction.

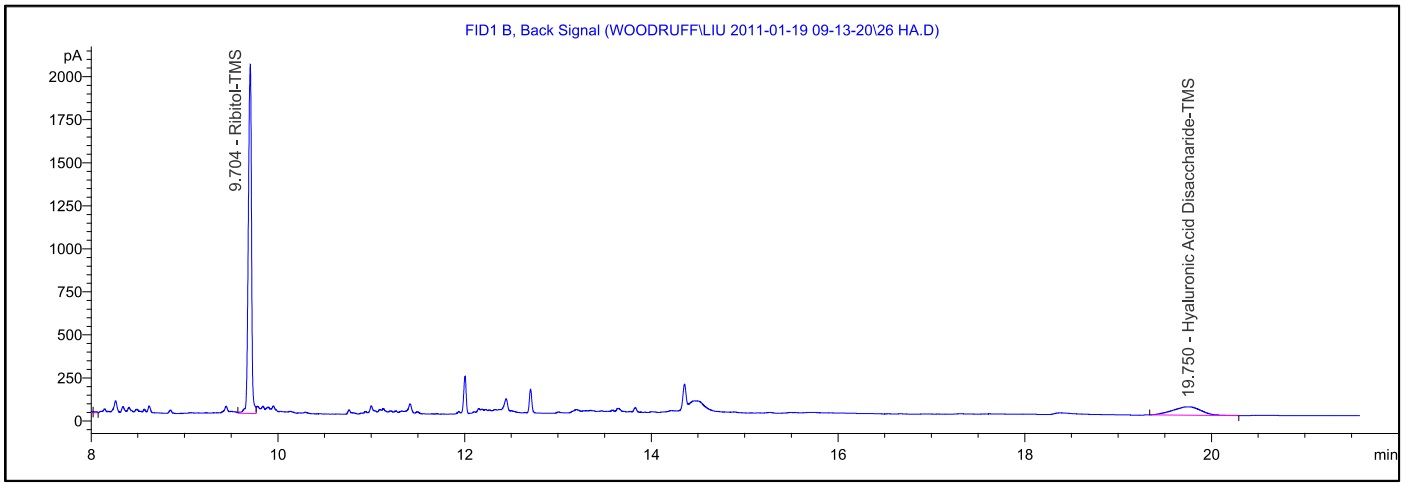



Figure 21: HA control sample ran on the flame GC/FID. The internal standard of ribitol is detected at 9.704 min and the wide peak of hyaluronic acid disaccharide appears around 19.750 min.

Even though the GC analysis of the supernatant looks resolute (Figure 20), the more sensitive GC/MS shows that cell wall components can obscure the internal standard (ribitol) peak (Figure 22).

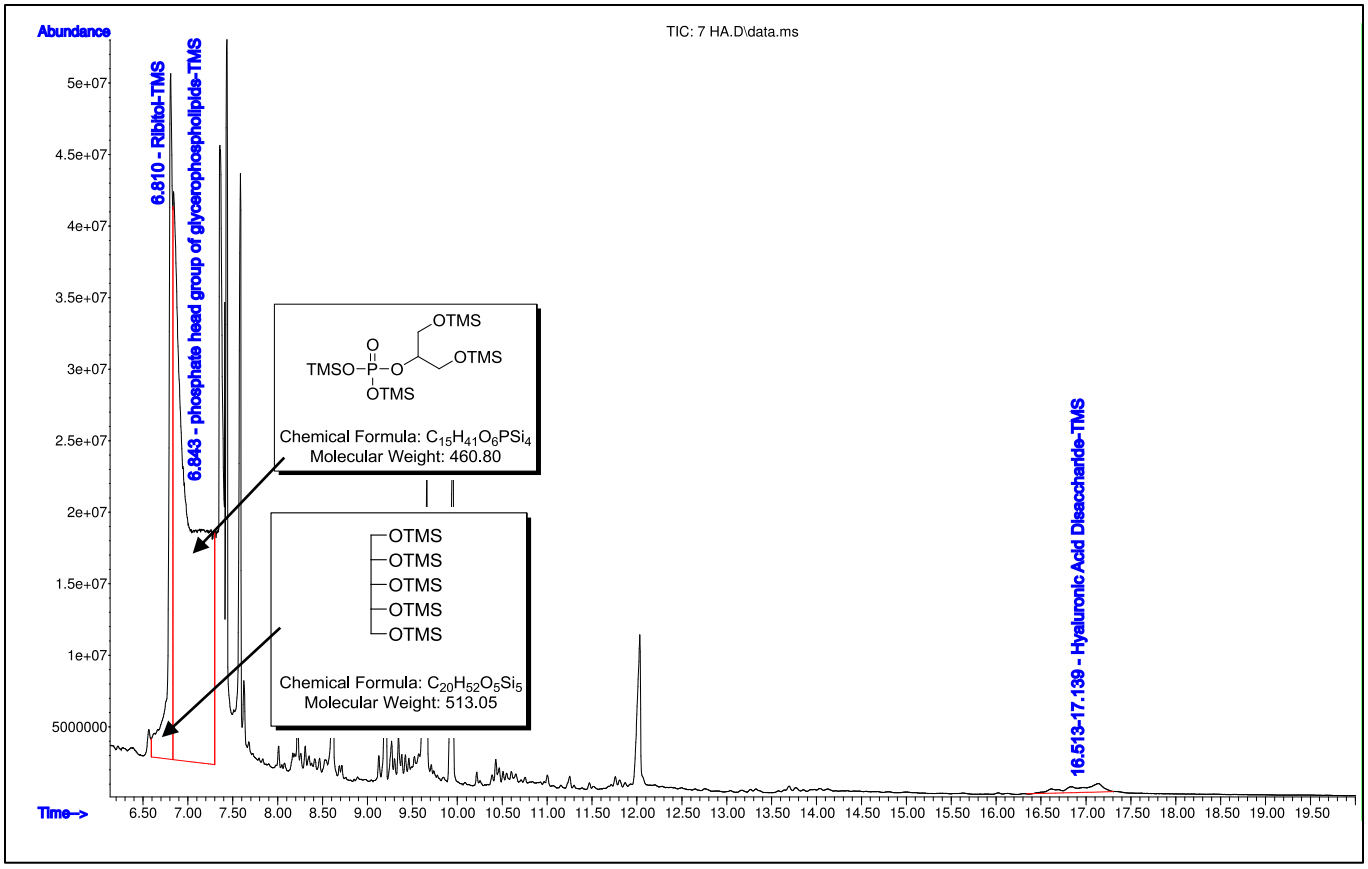



Figure 22: A chromatograph of supernatant at 60 minutes ran on the GC/MS. Using mass detector, cell wall components are found to overlap with the internal standard.

Mass spectroscopy confirms that the cell wall components actually overlapped significantly with ribitol. A majority of molecules around retention time 6.810 min is silylated ribitol (Appendix C, Figure C7), but an overlapping adjacent peak at 6.843 min is silylated phosphate heads of phospholipids (Appendix C, Figure C8).

As previously emphasized for the synthetic 18-sugar project, an overlap will interfere with the calculation of area ratio, which will make quantification inaccurate. We now know that ribitol should be avoided for any GC analysis of cellular solutions. For future cellular samples, myo-inositol should replace ribitol because it has a retention

time around 11.9 min on GC and 9.0 min on GC/MS, which are outside of the retention time range of most phospholipid derivatives.

# Conclusions

Through treating the dried carbohydrates with hydroxylamine hydrochloride, the carbonyl group can be converted to an open chain oxime. This step helps to stabilize the whole sugar through the subsequent silyl ether formation reaction with TMSI. The concentration procedure enhances the signal-to-noise ratio, especially for biological solutions. During the extraction, hexane is used to isolate the non-polar derivatives of carbohydrate while the saturated hydroxylamine solution helps to form a viscous layer of impurities. Comparing to other reagents, such as alcohols[3,7] or acetonitrile[3], an extraction step with hexane is best suited for cleaning up sensitive biological solutions because there is no evidence of physical reaction

The analysis of the synthetic mixture of 18 sugars is so far a qualitative project. All sugars separate distinctly and their chromatographs show resolute peaks with predictable retention times. Calibration data for each compound is in progress to allow for the calculation of the theoretical detection limits.

When analyzing rat urine, we are able to quantify D-mannitol, sucrose, sucralose, and lactulose by using ribitol as an internal standard. All of these sugars show resolute peaks and predictable retention times. Our qualitative and quantitative results have been supported by statistical analysis from The Ohio State Medical Center.

The cellular permeability project with hyaluronic acid disaccharide is on-going. Even though the hyaluronic acid disaccharide peaks are characteristically small and wide, they can be identified successfully by retention times. Other discoveries, such as an abundant amount and variety of cell wall component, are also valuable. We now

have plans for an improved internal standard for this study. Because the actual procedure was able to derive a distinct hyaluronic acid disaccharide peak, we hope to quantify this study with future samples.

# Future Work

Currently, we have completed rat urine analyses and have successfully separated 18 sugars in a synthetic mixture. In order to publish the information for the 18 sugar project, an internal standard that preferably gives a resolute and narrow peak should be used in order to make a calibration curve and to report the theoretical detection limit of each carbohydrate. We hope to publish this work in summer quarter. As for the cellular permeability project with hyaluronic acid disaccharide, ribitol is no longer the internal standard of choice because its peak overlaps with some cell wall components in the lysed cell samples. A new internal standard, myo-inositol, will replace ribitol when deriving the next batch of samples.

# Experimental

All reagents and solvents were obtained from Sigma Aldrich unless otherwise stated. D-mannitol, D-sucrose, D-sucralose, and D-lactulose for rat urine analysis were acquired from Acros-Organics. Eppendorf micropipettes (200 µL and 1000 µL) and glass micropipettes were used for transfers. The GC vials (screw top, 300 µL) were bought from MicroSolv Technology Corporation. The borosilicate culture tubes (12 x 75 mm) and all other glassware were obtained from Fisher Scientific. The evaporation apparatus was made in The Chemistry Department's machine shop (Figure 23). It contained a top portion that distributed nitrogen through 20 stainless steel tubes. The bottom portion was an aluminum heating block, which accommodated 20 culture tubes and contained a slot for the temperature-sensing probe of the Corning PC-420D Heater. This apparatus provided even heating and quick evaporation for a large quantity of samples.


Figure 23: Evaporation apparatus.

Two gas chromatographic equipment were bought from Agilent Technologies and were used in these projects.

**The GC system (7890A)** had a DB-5 column (5% phenyl methyl siloxane). This machine was used for the rat urine analysis project, which included finding the retention time of each carbohydrate, running the serial dilutions, and analyzing hundreds of actual urine samples. Also, it was used exclusively for analyzing synthetic mixtures of 18

sugars and was utilized to examine cellular and acellular samples with hyaluronic acid disaccharides.

The method on this GC system has an initial oven temperature of 100 ˚C. This temperature was first held for 3 min and then increased at a rate of 17 ˚C/min to a final temperature of 280 ˚C. The final temperature was held for 8 min to make a total of 21.588 min. The post run lasted 1 min at 100 ˚C and had a flow rate of 4 mL/min. The back nitrogen inlet was set on splitless mode with the heater at 250 ˚C, pressure at 8.5123 psi, and total flow at 20 mL/min. The septum purge flow and purge flow to split vent were set at 3 mL/min and 15 mL/min at 0.75 min respectively. The gas saver was on and flows at a rate of 20 mL/min after 2 min. The column (5% phenyl methyl siloxane: 958.74921) can tolerate a maxium temperature of 325 ˚C and has an initial dimension of 30 m x 320 µm x 0.25 µm (length x diameter x film thickness). However, the actual length of the column was less than 30 m, because its tip was cut off to reduce contamination when the column was installed. (This change in length did affect retention times but did not interfere with the order of separation or identification.) Within the column, the pressure, flow rate, average velocity, and holdup time were 10.815 psi, 2 mL/min, 37.143 cm/sec, and 1.3461 min respectively. The back injector, which consisted of a 10 µL syringe, was used. Two solvents, methanol (8 µL) and acetone (8 µL), were used as washes. There were two pre- and two post-injection washes for each solvent. The syringe was washed with 8 µL of sample, and injected (1 µL) after 6 pumps. The draw and dispense speeds were set at 300 µL/min and 6000 µL/min respectively. There was no injection dwell time (pre- or post-injection) or viscosity delay.

The back flame ionization detector (FID) was used. It burnt at a temperature of 250 ˚C with hydrogen (45 mL/min), air (450 mL/min), and makeup flow (43 ml/min).

**The GC/MS system (6850/5975C)** had a HP-5MS column (5% phenyl methyl siloxane). This machine was used in conjunction with the 7890A GC system to confirm the results for cellular and acellular samples with hyaluronic acid disaccharides.

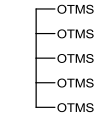
The method on this GC/MS system had a solvent delay (6 min). During a run, it had an initial oven temperature of 100 ˚C. This temperature was immediately increased at a rate of 17 ˚C/min to a final temperature of 270 ˚C. The final temperature was held for 20 min. The total running time was 30 min with 0 min and 0 ˚C of post time and temperature. The helium inlet was set on split mode with the initial temperature at 250 ˚C and pressure at 12.83 psi. It split ratio was 1:1 with a split flow of 1.2 mL/min and a total flow of 5.1 mL/min. The gas saver was on and flows at a rate of 20 mL/min after 2 min. The column (5% phenyl methyl siloxane: 19091S-433E) can tolerate a maxium temperature of 325 ˚C and has an initial dimension of 30 m x 250 µm x 0.25 µm (length x diameter x film thickness). However, the actual length of the column was less than 30 m, because its tip was cut off to reduce contamination when the column was installed. (This change in length did affect retention times but did not interfere with the order of separation or identification.) Within the column, the pressure, flow rate, and average velocity, and holdup time were 12.84 psi, 1.2 mL/min, and 41 cm/sec respectively. The front injector, which consisted of a 10 µL syringe, was used. Two solvents, methanol (8 µL) and acetone (8 µL), were used as washes. There were two pre- and two post-injection washes for each solvent. The syringe was washed with 8 µL of sample, and injected (1 µL) after 2 pumps. The plunger speed was set at fast. There was no injection

dwell time (pre- or post-injection) or viscosity delay. Detection was performed by mass spectroscopy (MS) at a temperature of 250 ˚C. The MS had a mass scan parameter between 40 and 700 and a threshold mass of 150.

General derivitization procedure:

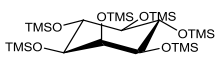
A solution of hydroxylamine hydrochloride in pyridine (10mg/mL, at least 5 equivalent of hydroxylamine hydrochloride per ketone and aldehyde) was added to dried carbohydrate. The sample was loosely covered with parafilm to allow slight evaporation of water. It was heated at 70 °C for 1 h and vortexed every 15 min. After, the sample was cooled to rt, 1-(trimethylsilyl)imidazole (TMSI, at least 40 equivalent of TMSI per nucleophile) was added. The sample was topped with nitrogen, sealed, and vortexed. It was heated at 70 °C for 30 min and vortexed every 10 min. The volume was reduced at 65 °C under a stream of nitrogen until it reached a syrup consistency. After the concentrated sample was cooled to rt, hexane (preferably 150 µL; 200 µL can also be used to facilitate difficult transfers) and a saturated solution of hydroxylamine hydrochloride in pyridine (50 µL; 100 µL should be used for sugar concentrations that are greater than 1g/L) were added to produce two distinct layers. The mixture was topped with nitrogen, vortexed, and centrifuged for 3-5 min at 2500 RPM. The supernatant (30 µL) was transferred to an insert within GC vial and evaluated by gas chromatography and/or gas chromatography and mass spectrometry (GC/MS) within 4 h.

# Analysis of Synthetic Mixture of 18 Sugars



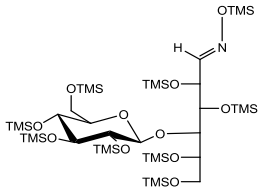

**1. Ribitol derivative**

Chemical Formula: $C_{20}H_{52}O_5Si_5$
Molecular Weight: 513.05



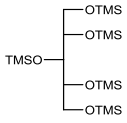

**7. Myo-inositol derivative**

Chemical Formula: $C_{24}H_{60}O_6Si_6$
Molecular Weight: 613.24



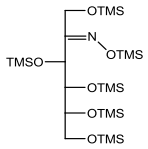

**13. D-cellobiose derivative**

Chemical Formula: $C_{39}H_{95}NO_{11}Si_9$
Molecular Weight: 1006.94



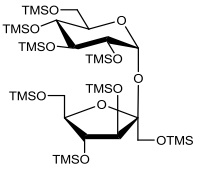

**2. Xylitol derivative**

Chemical Formula: $C_{20}H_{52}O_5Si_5$
Molecular Weight: 513.05



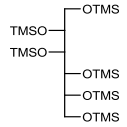

**8. D-fructose derivative**

Chemical Formula: $C_{24}H_{61}NO_6Si_6$
Molecular Weight: 628.26



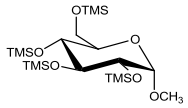

**14. D-sucrose derivative**

Chemical Formula: $C_{36}H_{86}O_{11}Si_8$
Molecular Weight: 919.75



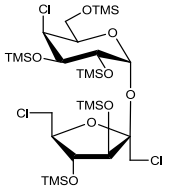

**3. D-mannitol derivative**

Chemical Formula: $C_{24}H_{62}O_6Si_6$
Molecular Weight: 615.26



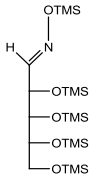

**9. α-D-methylglucoside derivative**

Chemical Formula: $C_{19}H_{46}O_6Si_4$
Molecular Weight: 482.91



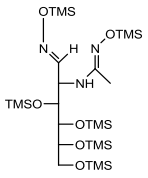

**15. D-sucralose derivative**

Chemical Formula: $C_{27}H_{59}Cl_3O_8Si_5$
Molecular Weight: 758.54



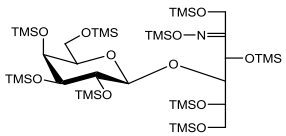

**4. D-ribose derivative**

Chemical Formula: $C_{20}H_{51}NO_5Si_5$
Molecular Weight: 526.05



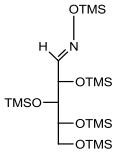

**10. N-acetyl-D-glucosamine derivative**

Chemical Formula: $C_{26}H_{65}N_3O_6Si_6$
Molecular Weight: 684.32



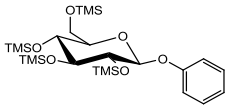

**16. D-lactulose derivative**

Chemical Formula: $C_{39}H_{95}NO_{11}Si_9$
Molecular Weight: 1006.94



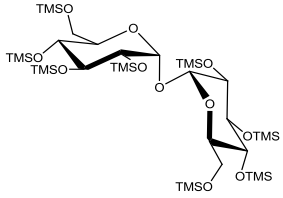

**5. D-xylose derivative**

Chemical Formula: $C_{20}H_{51}NO_5Si_5$
Molecular Weight: 526.05



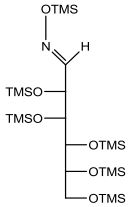

**11. Phenyl-β-D-glucopyranoside derivative**

Chemical Formula: $C_{24}H_{48}O_6Si_4$
Molecular Weight: 544.98



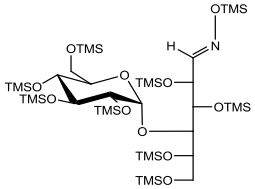

**17. D-trehalose dihydrate derivative**

Chemical Formula: $C_{36}H_{86}O_{11}Si_8$
Molecular Weight: 919.75



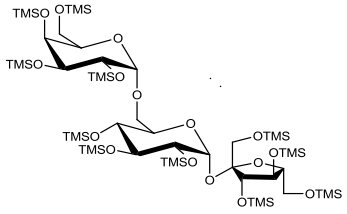

**6. D-mannose derivative**

Chemical Formula: $C_{24}H_{61}NO_6Si_6$
Molecular Weight: 628.26




**12. D-maltose derivative**

Chemical Formula: $C_{39}H_{95}NO_{11}Si_9$
Molecular Weight: 1006.94




**18. D-raffinose pentahydrate derivative**

Chemical Formula: $C_{51}H_{120}O_{16}Si_{11}$
Molecular Weight: 1298.43

Deriving single carbohydrates:

Each carbohydrate of sugars 1-18 (see figure 5) was derived separately to find its retention time. Carbohydrate/water solution (1 mL, 100 mg/L) was transferred into a culture tube. The solution was evaporated in an oven overnight at 65 ˚C. A 20 % hydroxylamine solution (50 µL, 20 mg hydroxylamine hydrochloride in 1 mL of pyridine) and additional pyridine (50 µL) were added to the sample. This solution was loosely covered with parafilm and heated at 70 ˚C for 1 h. It was vortexed every 15 min during heating. After the solution was cooled to rt, of 1-(trimethylsilyl)imidazole (100 µL) was added. The sample was immediately topped off with nitrogen and sealed with parafilm. It was heated again for 30 min at 70 ˚C and vortexed every 10 min. The volume was reduced at 65 °C under a stream of nitrogen until it reached a syrup consistency. After the concentrated sample was cooled to rt, hexane (150 µL) and a saturated solution of hydroxylamine hydrochloride in pyridine (50 µL) were added to produce two distinct layers. The mixture was topped with nitrogen, vortexed, and centrifuged for 4 min at 2500 RPM. The supernatant (50 µL) was transferred to a GC vial and evaluated by gas chromatography (7890A) within 4 h. The retention times of the derivatives were found to be: **1**. ribitol (9.694-9.739 min), **2**. xylitol (9.444-9.619 min), **3**. D-mannitol (10.763-11.021 min), **4**. D-ribose (10.050-10.099 min), **5**. D-xylose (9.886-9.927 min), **6**. D-mannose (first peak: 11.301-11.348 min, second peak: 11.422-11.498 min), **7**. myo-inositol (11.864-11.920 min), **8**. D-fructose (first peak: 11.034-11.072 min, second peak: 11.083-11.127 min), **9**. α-D-methylglucoside (10.643-10.683 min), **10**. N-acetyl-D-glucosamine (multiple peaks: 12.005-12.452 min), **11**.Phenyl-β-glucopyranoside (12.948-12.985 min), **12**. D-maltose (multiple peaks: 16.005-16.554), **13**. D-cellobiose

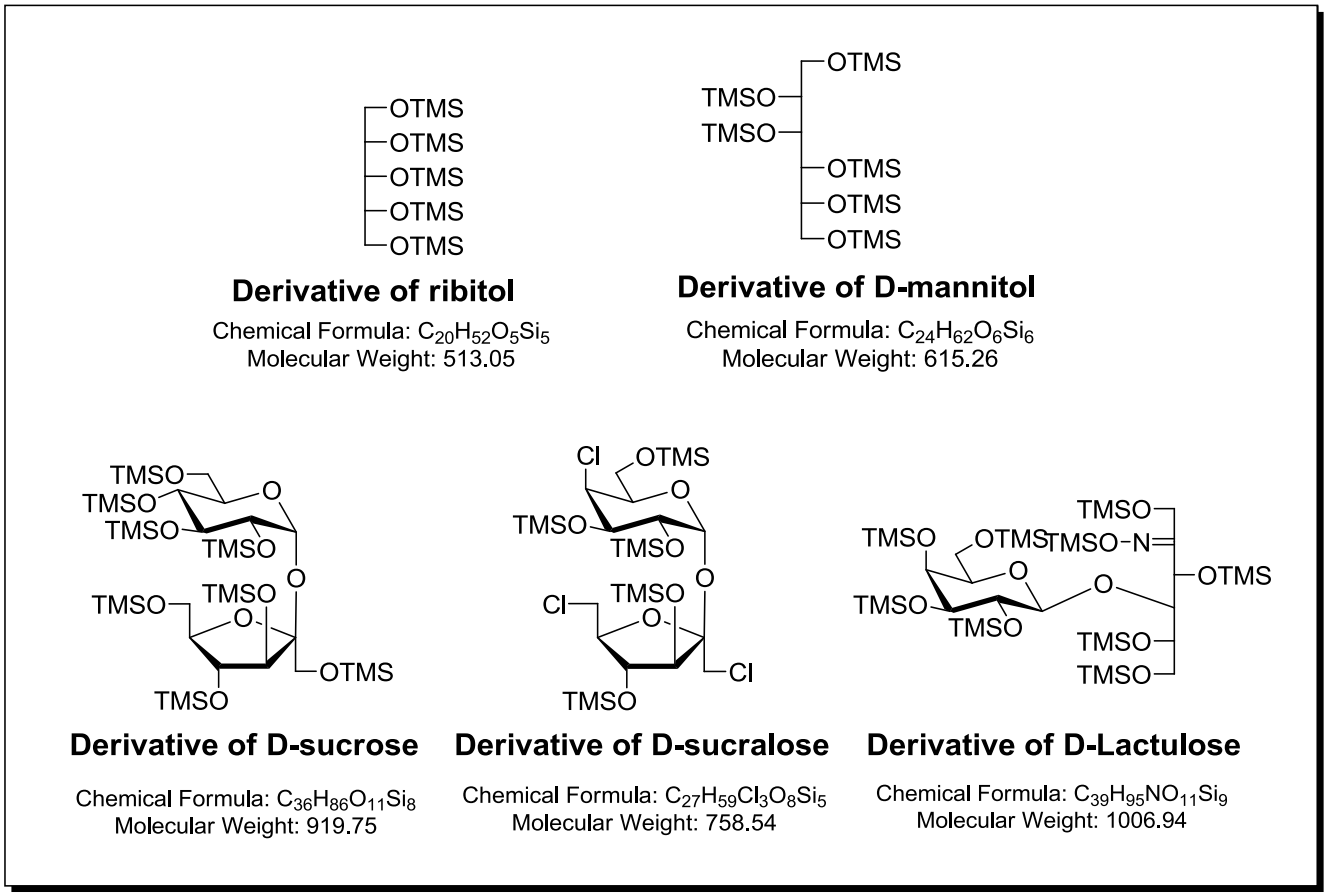
(15.788-15.893 min), **14**. D-sucrose (14.705-14.777 min), **15**. D-sucralose (15.148-15.219 min), **16**. D-lactulose (15.653-15.755 min), **17**. D-trehalose dihydrate (15.405-15.494 min), and **18**. D-raffinose pentahydrate (wide peak around 17.224-17.569 min).

Deriving the synthetic 18-sugar mixture:

200 mg of the 18 sugars of interest (**1**. Ribitol, **2**. xylitol, **3**. D-mannitol, **4**. D-ribose, **5**. D-xylose, **6**. D-mannose, **7**. myo-inositol, **8**. D-fructose, **9**. α-D-methylglucoside, **10**. N-acetyl-D-glucosamine, **11**.Phenyl-β-glucopyranoside, **12**. D-maltose, **13**. D-cellobiose, **14**. D-sucrose, **15**. D-sucralose, **16**. D-lactulose, **17**. D-trehalose dihydrate, and **18**. D-raffinose pentahydrate) were added to a 1 L volumetric flask with thymol (20 mg). The flask was filled to the 1 L mark with distilled water and the mixture was diluted to (1) 200 mg/L, (2) 100 mg/L, (3) 50 mg/L, (4) 25 mg/L, (5) 12.5 mg/L, (6) 6.25 mg/L, (7) 3.125 mg/L, (8) 1.56 mg/L, (9) 0.78 mg/L, and (10) 0.39 mg/L. 1 mL of each solution was transferred into a culture tube labeled with its corresponding dilution number (1-10) and concentration. The samples were evaporated in an oven overnight at 65 ˚C. A 20 % hydroxylamine solution (250 µL, 20 mg hydroxylamine hydrochloride in 1 mL of pyridine) was added to each sample. This solution was heated uncovered at 70 ˚C for 1 h. It was vortexed every 15 min during heating. After the solutions were cooled to rt, 1-(trimethylsilyl)imidazole (800 µL) was added. Immediately afterwards, the samples were topped off with nitrogen and sealed with parafilm. They were heated again for 30 min at 70 ˚C and vortexed every 10 min. Their volumes were reduced at 65 °C under a stream of nitrogen until they reached a syrup consistency. After the concentrated samples were cooled to rt, hexane (150 µL) and a saturated

solution of hydroxylamine hydrochloride in pyridine (100 µL) were added to produce two distinct layers. The mixtures were topped with nitrogen, vortexed, and centrifuged for 5 min. The supernatant (50 µL) was transferred to a GC vial and evaluated by gas chromatography (7890A) within 4 h. Each sugar's theoretical detection limits should be between 0 ng/mL and 25 ng/mL. (Note: The sugars were heated in hydroxylamine hydrochloride uncovered in this procedure to allow the evaporation of water. This is possible because these particular samples have large volumes of solvent.)

**Rat Urine Analysis**



Deriving single carbohydrates:

Each sugar was derived by itself to find its retention time. The sugar (10 mg) was added to a 100 mL volumetric flask with a drop of thymol dissolved in isopropyl alcohol. The flask was filled to the 100 mL mark with distilled water. This solution (1 mL) was transferred into a culture tube. The solution was evaporated in an oven overnight at 65 ˚C. A 20 % hydroxylamine solution (25 µL, 20 mg hydroxylamine hydrochloride in 1 mL of pyridine) and additional pyridine (75 µL) were added to the sample. This solution was loosely covered with parafilm and heated at 70 ˚C for 1 h. It was vortexed every 15 min during heating. After the solution was cooled to rt, of 1-(trimethylsilyl)imidazole (50 µL) was added. The sample was immediately topped off with nitrogen and sealed with parafilm. It was heated again for 30 min at 70 ˚C and vortexed every 10 min. The volume was reduced at 65 °C under a stream of nitrogen until it reached a syrup consistency. After the concentrated sample was cooled to rt, hexane (200 µL) and a saturated solution of hydroxylamine hydrochloride in pyridine (50 µL) were added to produce two distinct layers. The mixture was topped with nitrogen, vortexed, and centrifuged for 3 min at 2500 RPM. The supernatant (50 mL) was transferred to a GC vial and evaluated by gas chromatography (7890A) within 4 h. The retention times of the derivatives of ribitol, D-mannitol, D-sucrose, D-sucralose, and D-lactulose were found to be 7.406-7.527 min, 8.832-8.954 min, 12.960-13.135 min, 13.405-13.606 min, and 13.964-14.187 min.

Serial Dilution of carbohydrates of interest:

Ribitol, mannitol, sucrose, sucralose, and lactulose (1 g each) were placed in 20 mL scintillation vials. The open vials were stored in a desiccator at rt for 1 week befor use. The four sugars of interest (D-mannitol, D-sucrose, D-sucralose, and D-lactulose, 200 mg each) were added to a Class A, 1 L volumetric flask. A preservative solution, thymol in isopropanol (1 mL, 25 mg/mL), was also added. The flask was filled to the 1000 mL mark with distilled water and the mixture was serially diluted using 50 mL Class A volumetric flasks to produced nine 25 mL solutions of concentrations: (1) 200 mg/L, (2) 100 mg/L, (3) 50 mg/L, (4) 25 mg/L, (5) 12.5 mg/L, (6) 6.25 mg/L, (7) 3.125 mg/L, (8) 1.56 mg/L, and (9) 0.78 mg/L. Each of the 25 mL solutions were spiked using a calibrated pipette with a ribitol internal standard solution (500 µL, 5.100 g/L in $H_2O$) to a final ribitol concentration of 100 mg/L. The new concentrations of sugars of interest in these solutions are (1) 196.08 mg/L, (2) 98.04 mg/L, (3) 49.02 mg/L, (4) 24.51 mg/L, (5) 12.25 mg/L, (6) 6.13 mg/L, (7) 3.06 mg/L, (8) 1.53 mg/L, and (9) 0.77 mg/L (Figure 2).The solutions were refrigerated when they were not in use. 750 µL of each solution was transferred into a culture tube labeled with its corresponding dilution number (1-9). The samples were evaporated in an oven with desiccant overnight at 65 ˚C. A 20 % hydroxylamine solution (50 µL, 20 mg hydroxylamine hydrochloride in 1 mL of pyridine) and additional pyridine (50 µL) were added to each sample. This solution were sealed with parafilm and heated at 65 ˚C for 1 h. It was vortexed every 15 min during heating. After the solutions were cooled to rt, 1-(trimethylsilyl)imidazole (100 µL) was added to each tube. Immediately afterwards, the samples were topped off with nitrogen and sealed with parafilm. They were heated again for 30 min at 65 ˚C and vortexed every 10

min. Their volumes were reduced at 65 °C under a stream of nitrogen until they reached a syrup consistency (roughly 20 min). After the concentrated samples were cooled to rt, hexane (200 µL) and a saturated solution of hydroxylamine hydrochloride in pyridine (75 µL) were added in the preceding order to produce two distinct layers. The mixtures were topped with nitrogen, vortexed, and centrifuged for 3 min at 2500 RPM. The supernatant (50 µL) was transferred to a GC vial and evaluated by gas chromatography (7890A) within 4 h.

Construction of calibration curves:

Calibration curves were created using the serial dilutions and the equation: $y = mx + b$, where x is the concentration ratio of sugar of interest over internal standard and y is the ratio of the area under the peak of the sugar of interest to that of the internal standard (see Appendix B, Figure B3). The plots of the calibration curves were number from 1 to 9 representing the dilution numbers and their corresponding concentrations. A correlation value, which evaluated the amount of deviation of the plots from the actual curve with a value between -1 and 1, was automatically calculated by the Agilent software. The theoretical detection limits of the sugars of interest were determined by the x-intercept values: mannitol: 0.0418 mg/L, sucrose: 4.4717 mg/L, sucralose: 3.2883 mg/L, and lactulose: 1.7968 mg/L.

Preparation and derivatization of rat urine:

Frozen urine samples from *Rattus norvegicus* ranging in volume from 1.5 – 2.0 mL were delivered from The Ohio State Medical Center. Due to the special diets of

these rats, their urine might contain various concentrations of mannitol, sucrose, sucralose, and/or lactulose. The samples were thawed to rt and vortexed. An aliquot (1000 µL) were transferred to a culture tube using a calibrated pipette. Each urine sample was spiked with an internal standard solution of ribitol in water (100µL, 1.100 g/L) to a total ribitol concentration of 100 mg/L. The sample was vortexed, sealed, and centrifuged for 3 min at 2500 RPM. The supernatant (750 mL) was removed, transferred to a fresh culture tube, and evaporated to dryness at 65°C under a stream of nitrogen. This sample was cooled to rt and was ready for derivatization.

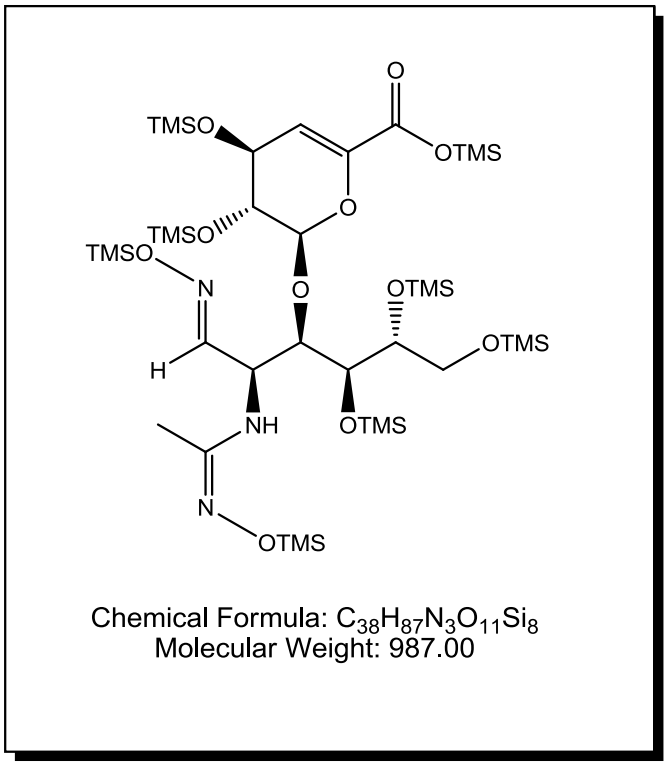
Derivatization of rat urine:

A 20 % hydroxylamine solution (50 µL, 20 mg hydroxylamine hydrochloride in 1 mL of pyridine) and additional pyridine (50 µL) were added to the sample. This solution was loosely covered with parafilm and heated at 65 ˚C for 1 h. It was vortexed every 15 min during heating. After the solution was cooled to rt, of 1-(trimethylsilyl)imidazole (100 µL) was added. The sample was immediately topped off with nitrogen and sealed with parafilm. It was heated again for 30 min at 65 ˚C and vortexed every 10 min. The parafilm was removed and the volume was reduced at 65 °C under a stream of nitrogen until the sample reached a syrup consistency. After the concentrated sample was cooled to rt, hexane (150 µL) and a saturated solution of hydroxylamine hydrochloride in pyridine (75 µL) were added to produce two distinct layers. The mixture was topped with nitrogen, vortexed, and centrifuged for 3 min at 2500 RPM. The supernatant (30 µL) was transferred to a GC vial and evaluated by gas chromatography system (7890A) within 4 h.

Quantitative and qualitative analysis:

The presence of sugars of interest within the urine is found with retention times. To quantify, the GC data from actual rat urine samples were put in an [(sugar of interest) / (internal standard)] ratio. Using this area ratio and the calibration curve, the concentration ratio of each of the sugar of interest were extrapolated and the approximate concentration was derived with this ratio (see Appendix B, Figure B3). With known retention times and area ratios, hundreds of rat urine samples were analyzed.

**Cellular Permeability of Hyaluronic Acid Disaccharide**




Chemical Formula: $C_{38}H_{87}N_3O_{11}Si_8$
Molecular Weight: 987.00

Deriving hyaluronic acid disaccharide:

The 18 sugars of interest (1 mg each) were added to a 10 mL volumetric flask with thymol (0.2 mg). The flask was filled to the 10 mL mark with distilled water and the

mixture was diluted to (1) 100 mg/L, (2) 20 mg/L, (3) 10 mg/L, (4) 5 mg/L, and (5) 2.5 mg/L. 1 mL of each solution was transferred into a culture tube labeled with its corresponding dilution number (1-5) and concentration. The samples were evaporated under a stream of nitrogen at 40 ˚C. 100 µL of a 20 % hydroxylamine solution (20 mg hydroxylamine hydrochloride in 1 mL of pyridine) was added to each sample. These solutions were loosely covered with parafilm and heated at 70 ˚C for 1 h. It was vortexed every 15 min during heating. After the solutions were cooled to rt, 1-(trimethylsilyl)imidazole (100 µL) was added. Immediately afterwards, the samples were topped off with nitrogen and sealed with parafilm. They were heated again for 30 min at 70 ˚C and vortexed every 10 min. Their volumes were reduced at 55 °C under a stream of nitrogen for 30 min. After the concentrated samples were cooled to rt, hexane (150 µL) and a saturated solution of hydroxylamine hydrochloride in pyridine (50 µL) were added to produce two distinct layers. The mixtures were topped with nitrogen, vortexed, and centrifuged for 4 min at 2500 RPM. The supernatant (30 µL) was transferred to a GC vial and evaluated by GC/MS (6850/5975C) within 4 h. (Note: The sugars were generally heated at a lower temperature because the solvent level was relatively low and the disaccharide could decompose at high temperatures.)

Deriving cellular solutions and supernatants:

A batch of samples (about 0.5 µL per each) were obtained in 1.5 mL microcentrifuge tubes. Each sample (400 µL) was transferred to a culture tube and spiked with of an internal standard solution of ribitol in distilled water (40 µL, 110mg/mL). The sample was vortexed and evaporated to dryness at 60 °C under a

stream of nitrogen. A 20 % hydroxylamine solution (50 µL, 20 mg hydroxylamine hydrochloride in 1 mL of pyridine) and additional pyridine (50 µL) were added to each sample. These solutions were seal with parafilm and heated at 60 ˚C for 1 h. It was vortexed every 15 min during heating. The samples were uncovered afterwards and heated for 20 min at 65 ˚C. After the solutions were cooled to rt, 1-(trimethylsilyl)imidazole (200 µL) was added. Immediately afterwards, the samples were topped off with nitrogen and sealed with parafilm. They were heated again for 40 min at 65 ˚C and vortexed every 10 min. Their volumes were reduced at 55 °C under a stream of nitrogen for 90 min. After the concentrated samples were cooled to rt, hexane (150 µL) and a saturated solution of hydroxylamine hydrochloride in pyridine (50 µL) were added to produce two distinct layers. The mixtures were topped with nitrogen, vortexed, and centrifuged for 4 min at 2500 RPM. The supernatant (30 µL) was transferred to a GC vial and evaluated by GC (7890A) and GC/MS (6850/5975C) within 4 h. (Note: The sugars were generally heated at a lower temperature for a longer period of time. This is because the solvent level was relatively low and the disaccharide could decompose at high temperatures.)

## References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# Appendix A

## Gas Chromatographs of Synthetic mixture of 18 sugars

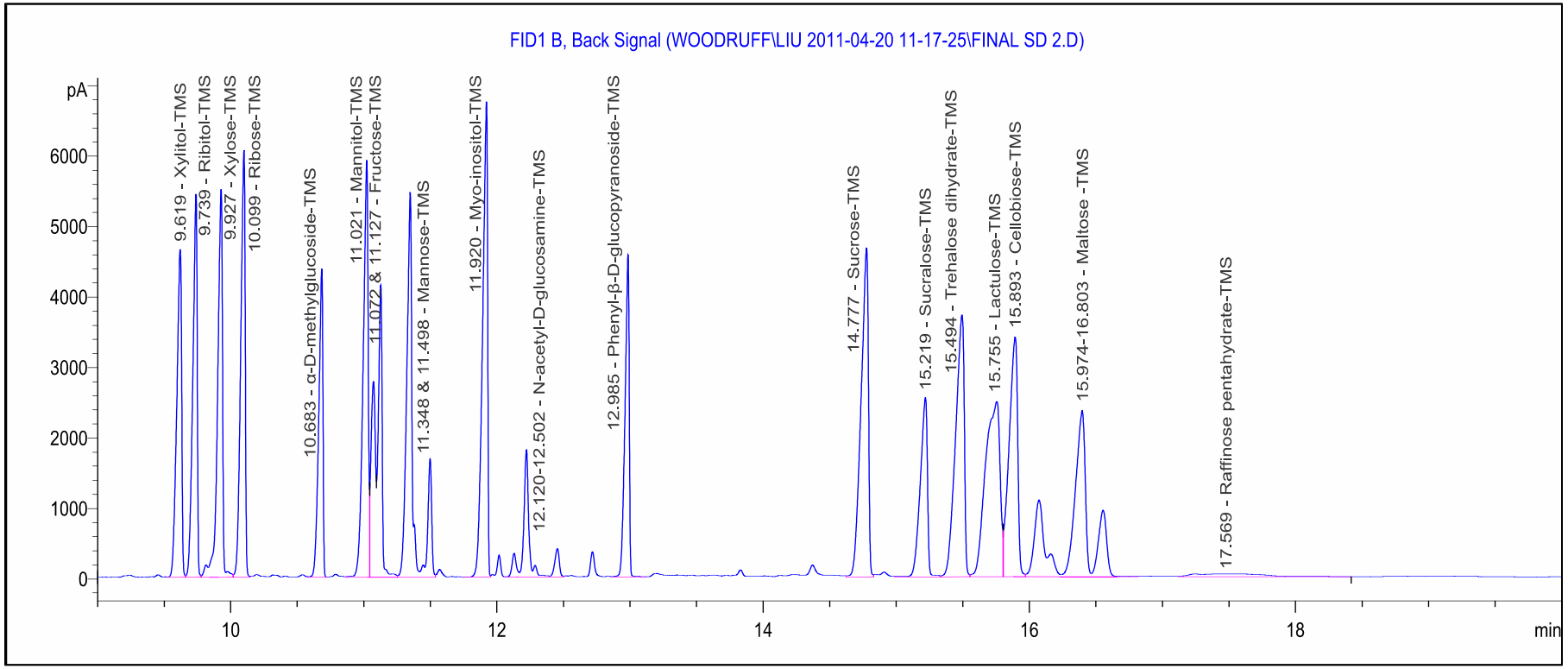



Figure A1: Chromatograph of all 18 sugars at equal concentrations of 50 mg/L.

# Appendix B

## Gas Chromatographs of Rat Urine

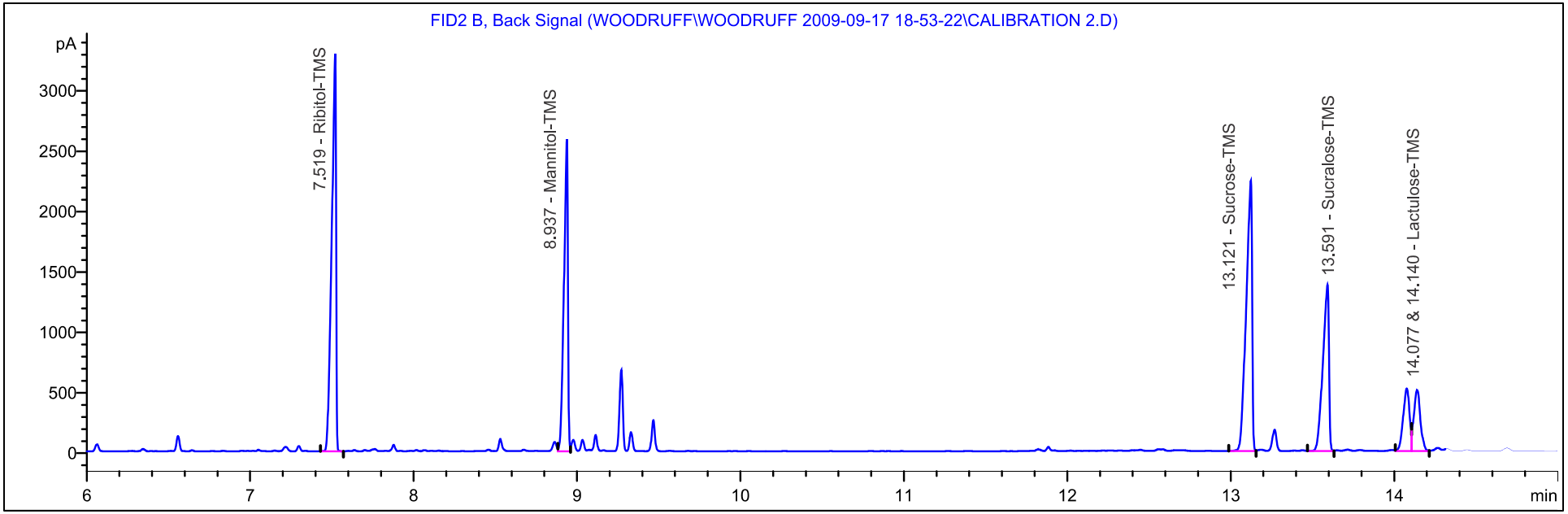



Figure B1: All five carbohydrate probes in a synthetic mixture (100 mg/L per sugar).

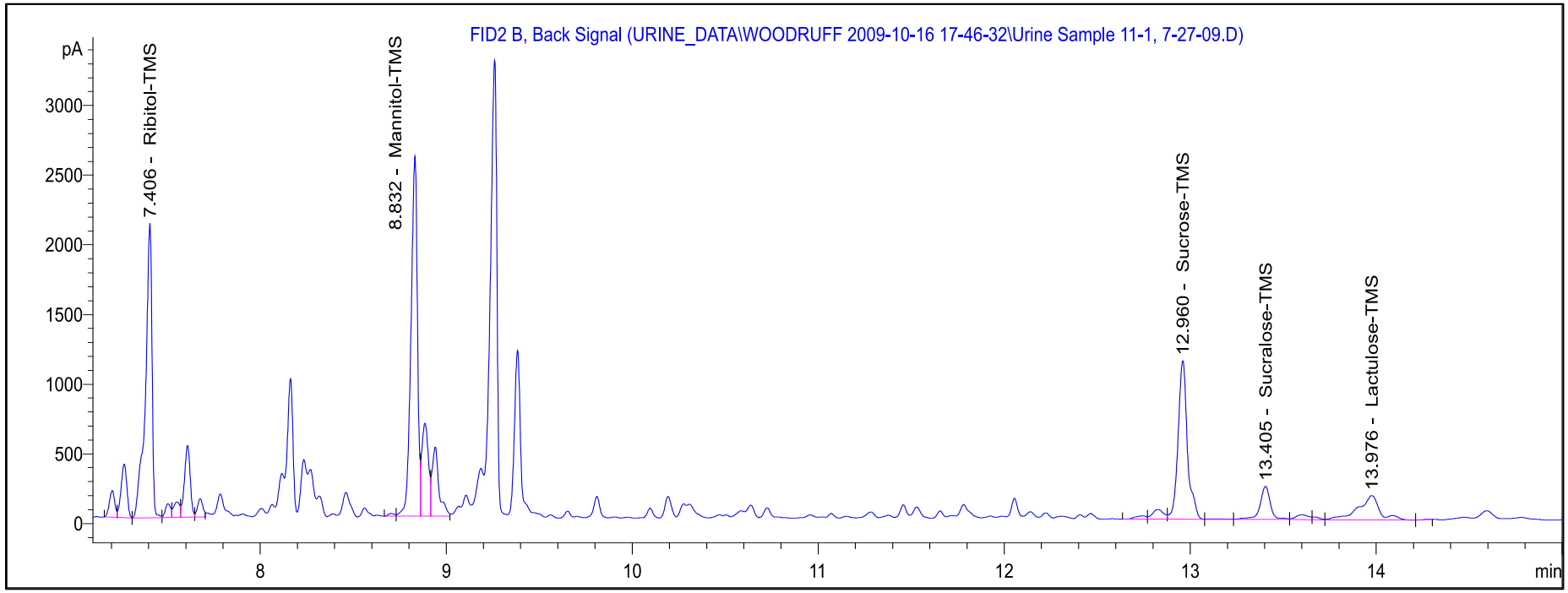
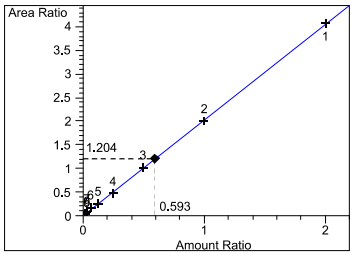
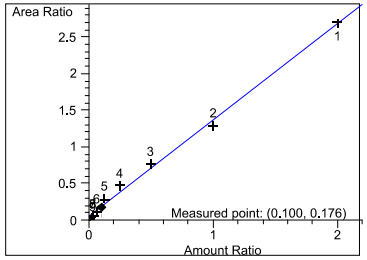


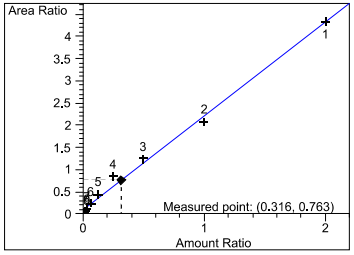

Figure B2: Urine analysis for sugar identification and concentration. This procedure is able to gently clean up the background noises of a complex body fluid, such as urine, while avoid diminishing the signal of carbohydrates.



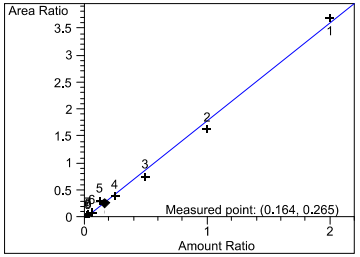

```
Mannitol-TMS at exp. RT: 8.807
FID2 B, Back Signal
Correlation:          0.99989
Residual Std. Dev.:   0.02085
Formula: y = mx + b
      m:      2.02723
      b:      8.47866e-4
      x: Amount
      y: Area
```




```
Sucralose-TMS at exp. RT: 13.393
FID2 B, Back Signal
Correlation:          0.99771
Residual Std. Dev.:   0.06131
Formula: y = mx + b
      m:      1.32180
      b:      4.34641e-2
      x: Amount
      y: Area
```




```
Sucrose-TMS at exp. RT: 12.918
FID2 B, Back Signal
Correlation:          0.99684
Residual Std. Dev.:   0.11509
Formula: y = mx + b
      m:      2.11227
      b:      9.44541e-2
      x: Amount
      y: Area
```




```
Lactulose-TMS at exp. RT: 14.002
FID2 B, Back Signal
Correlation:          0.99731
Residual Std. Dev.:   0.09096
Formula: y = mx + b
      m:      1.81154
      b:     -3.25504e-2
      x: Amount
      y: Area
```

Figure B3: Calibration curves for the carbohydrates of interests. These curves (in blue) are composed from nine 2-fold serial dilutions of a synthetic mixture. The serial dilutions start with a concentration of 196 mg/L for dilution number 1. Notice the correlation values for these calibration curves are above 0.99. This extremely high value shows that the derivatization procedure can be applied across a wide range of concentrations (from 196 mg/L to 0.77 mg/L) and still develop valid results. Also, the concentrations of all 4 sugars of interest can be extrapolated from these calibration curves using the (sugar of interest) / (internal standard) ratio.

# Appendix C

## Gas Chromatographs of the Cellular Solutions with Hyaluronic Acid

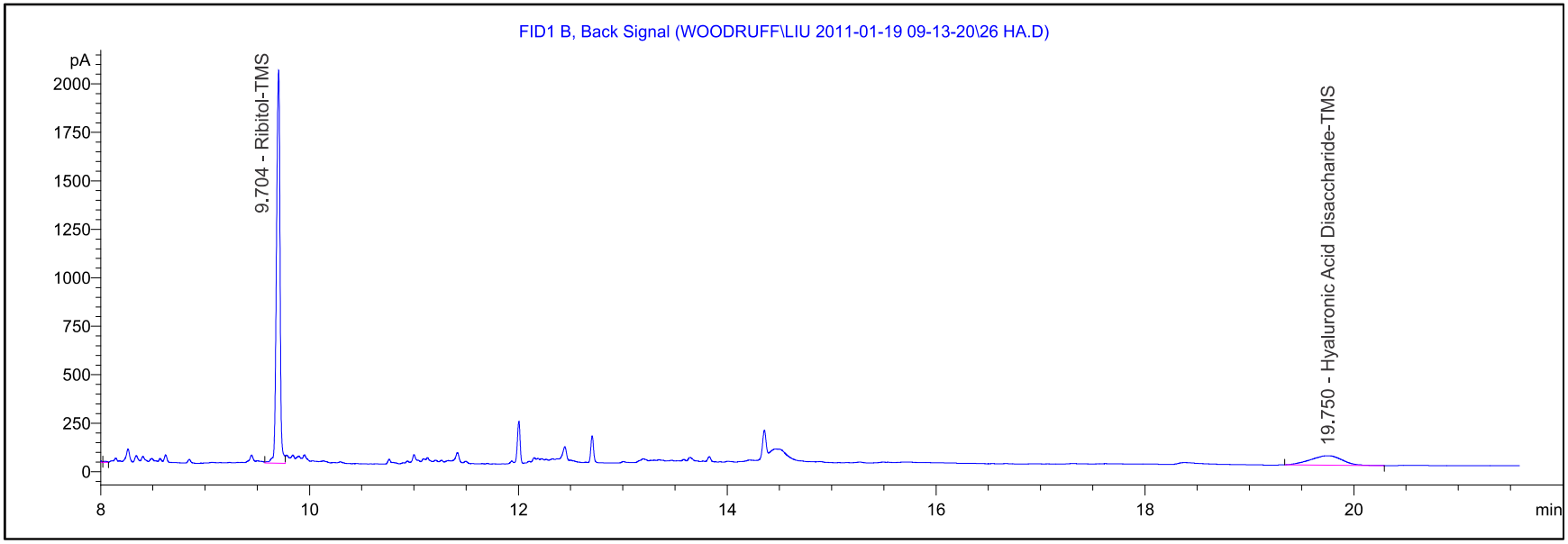



Figure C1: Control sample ran on the FID-GC 7890A. The internal standard of ribitol is located at 9.704 min, and the wide peak of hyaluronic acid disaccharid appears around 19.750 min.

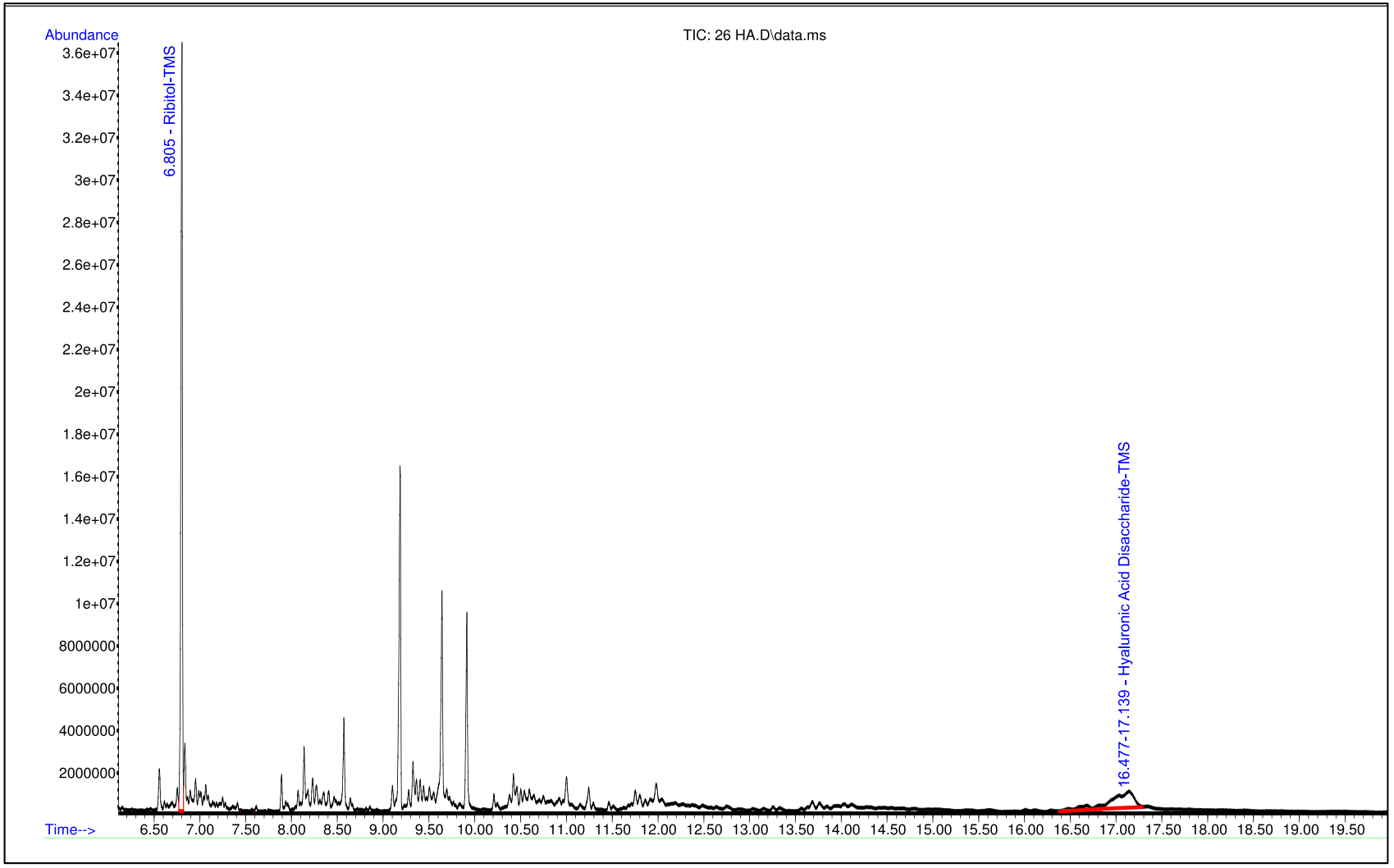



Figure C2: Control sample as in figure C1 ran on the GC/MS. The internal standard of ribitol appears at 6.805 min, and the wide peak of hyaluronic acid is located at 17.145 min. The significant amount of noise is caused by the high sensitivity, prolonged usage, and thus contamination of the column. However, this GC/MS is still very valuable for identifying molecules of interest and types of contaminations.

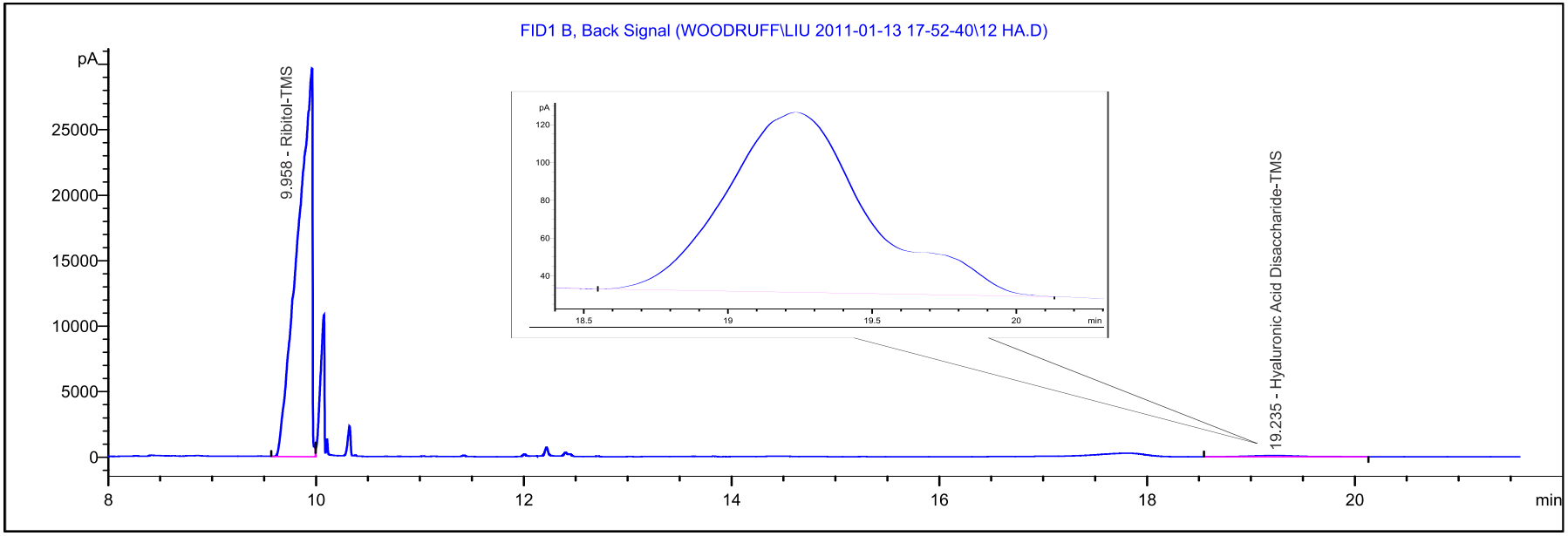



Figure C3: Lysed cells at 60 minutes ran on the GC. The zoomed-in Hyaluronic acid disaccharide peak occurs at 19.235 min.

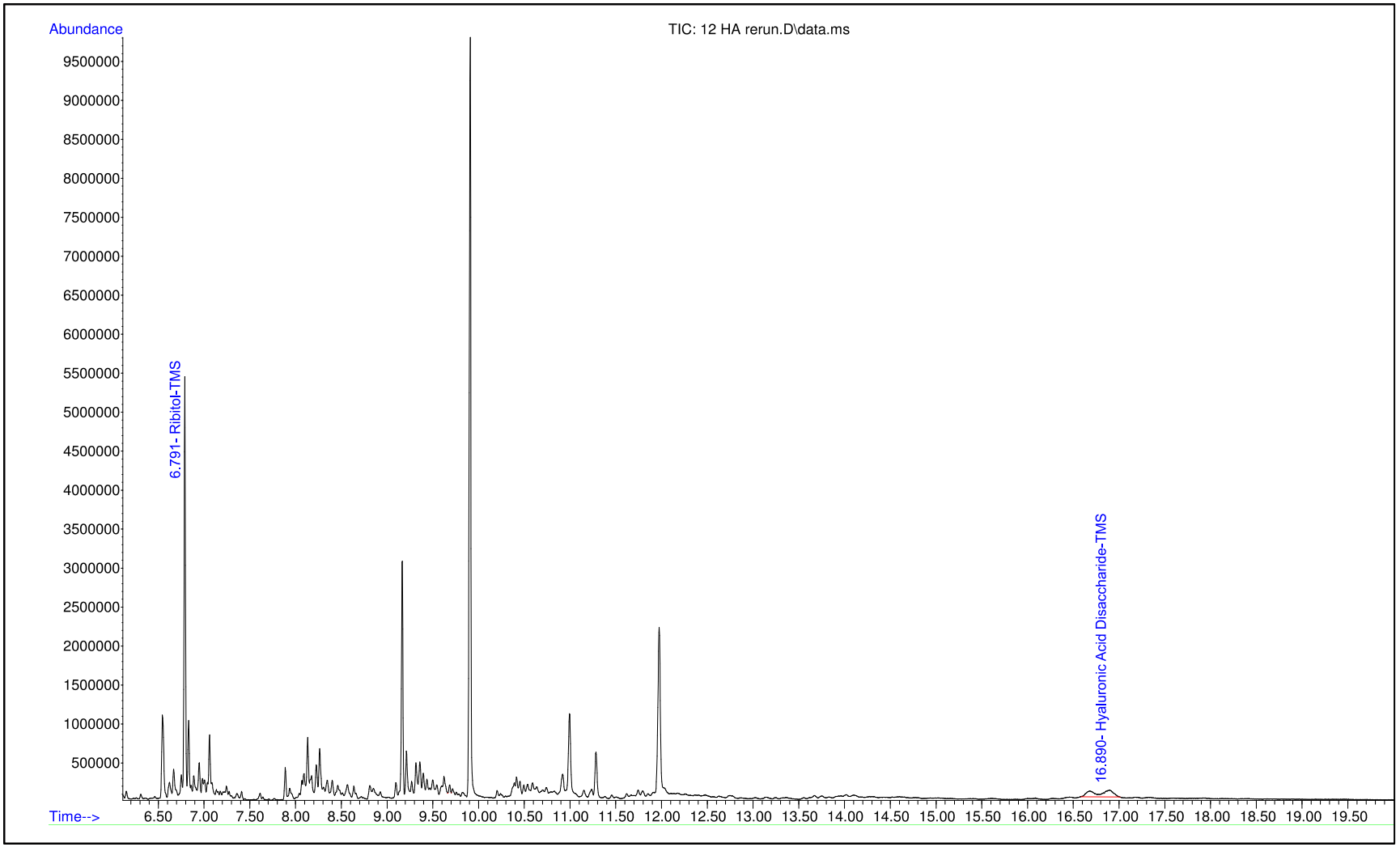



Figure C4: Lysed cells as in figure C3 at 60 minutes ran on the GC/MS (6850/5975C). (Note: This sample is slightly more dilute and was run after 24 h. Samples with a low or overlapped standard peak like this one at 6.791, are usually discarded because its analysis would be unreliable.)

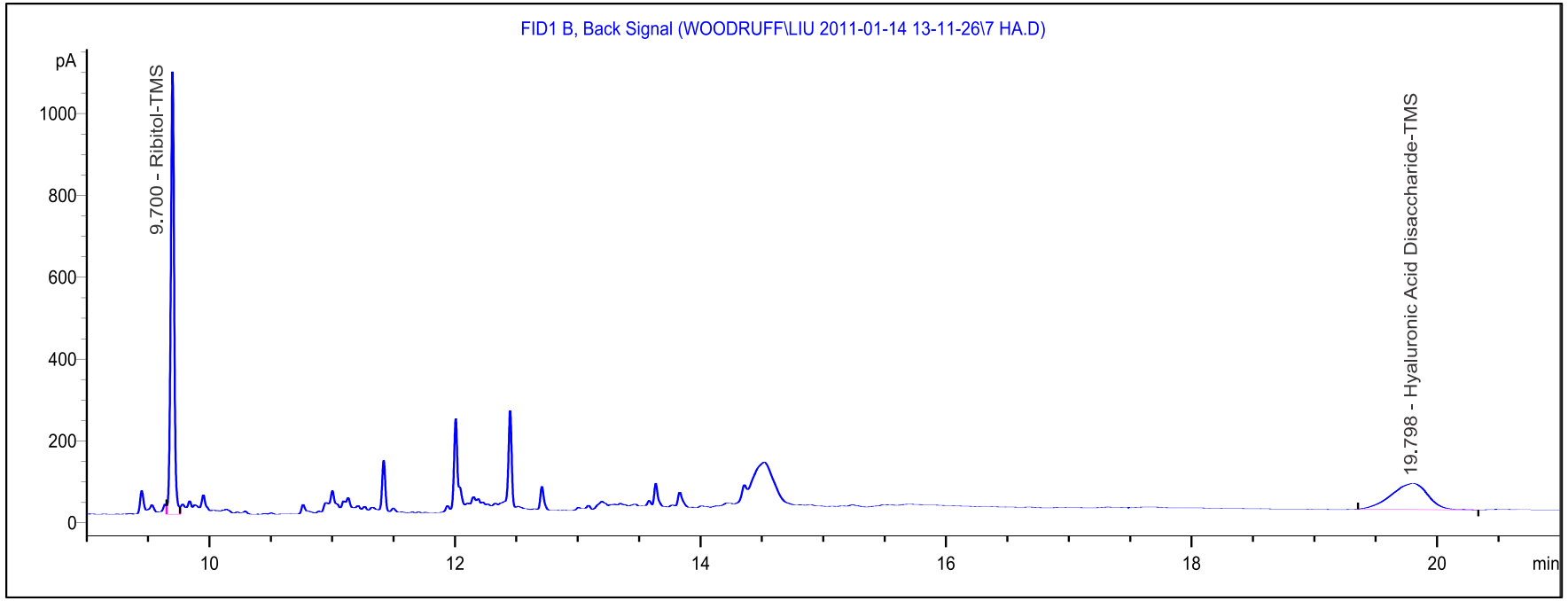



Figure C5: Supernatant at 60 minutes ran on the GC. The small amount absorbed by the cell can be derived from the concentration of hyaluronic acid in the supernatant.

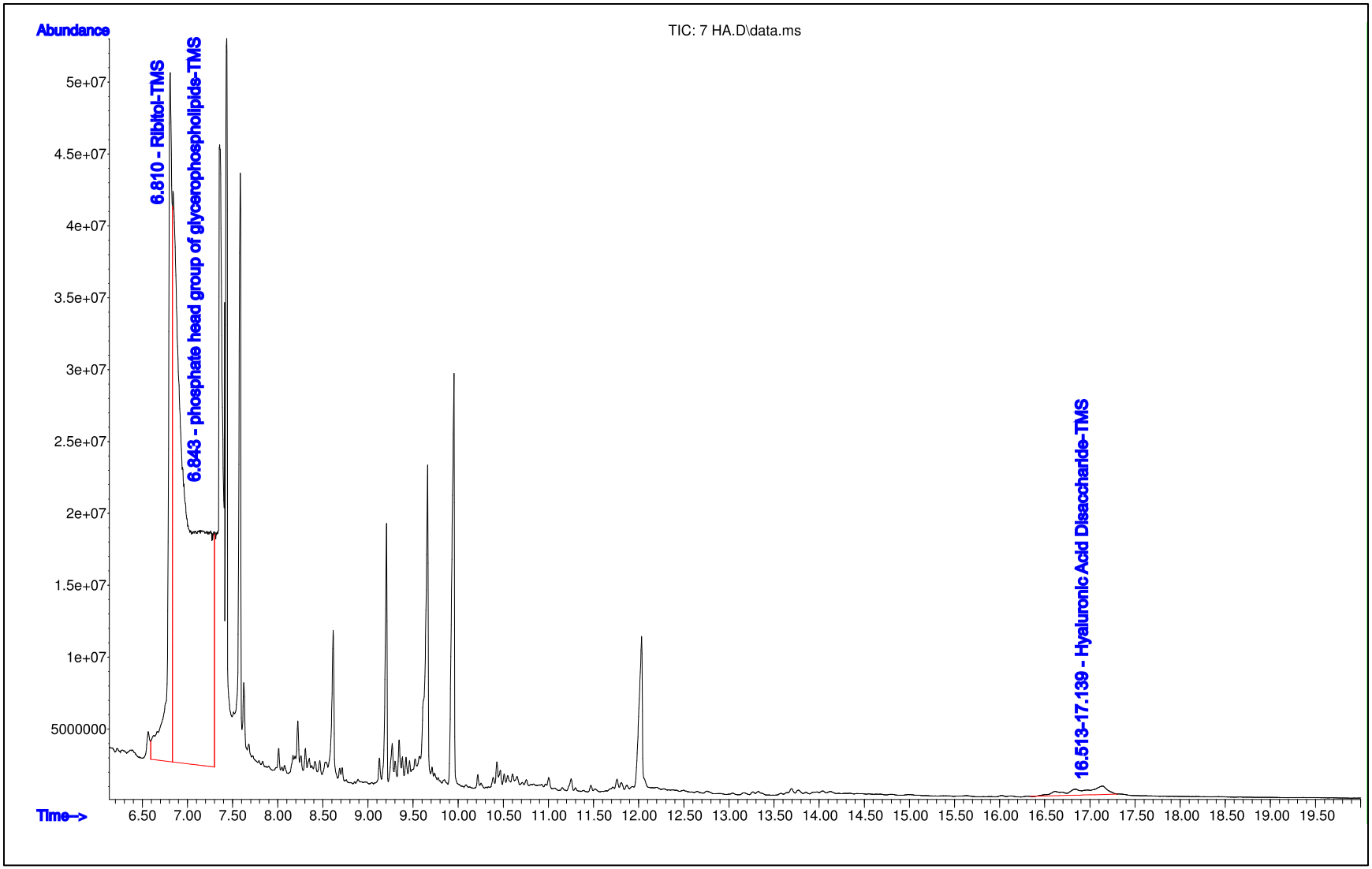



Figure C6: Supernatant sample as in Figure C5 ran on GC/MS. Using mass spectroscopy on this more sensitive machine, cell wall components are found to overlap with the ribitol peak. Because this overlap obscures the actual area under the ribitol peak, it can interfere with the quantification of hyaluronic acid disaccharide

.

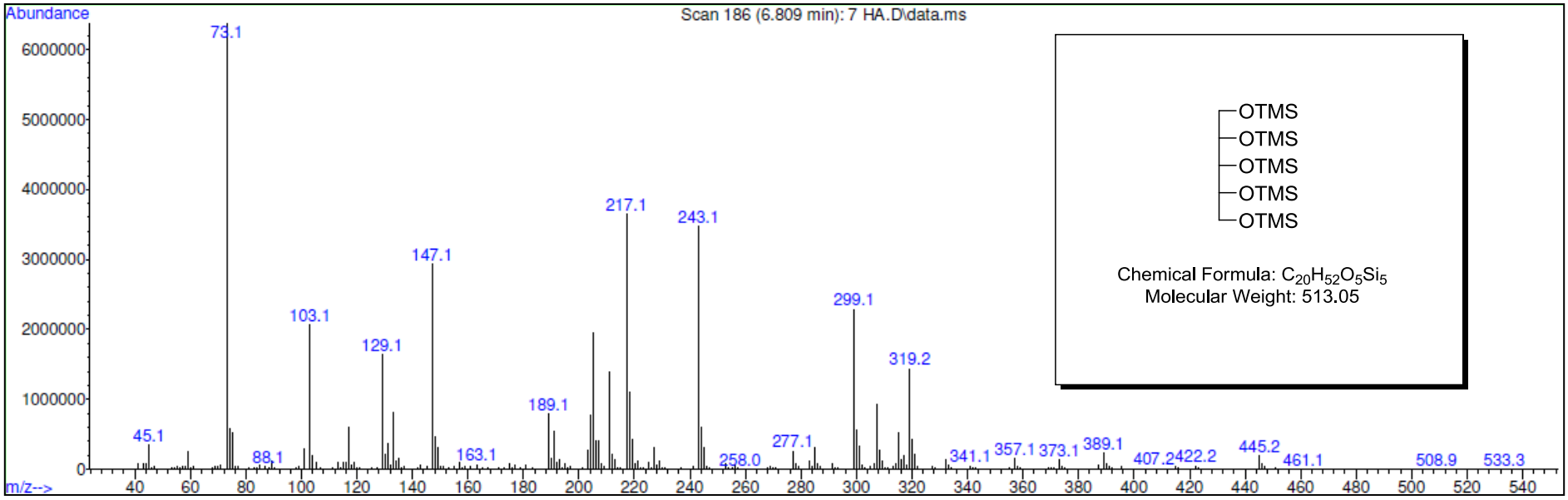



Figure C7: Mass spectroscopic analysis of the molecule that emerged at 6.809 min on figure C6. This peak contains mostly ribitol.

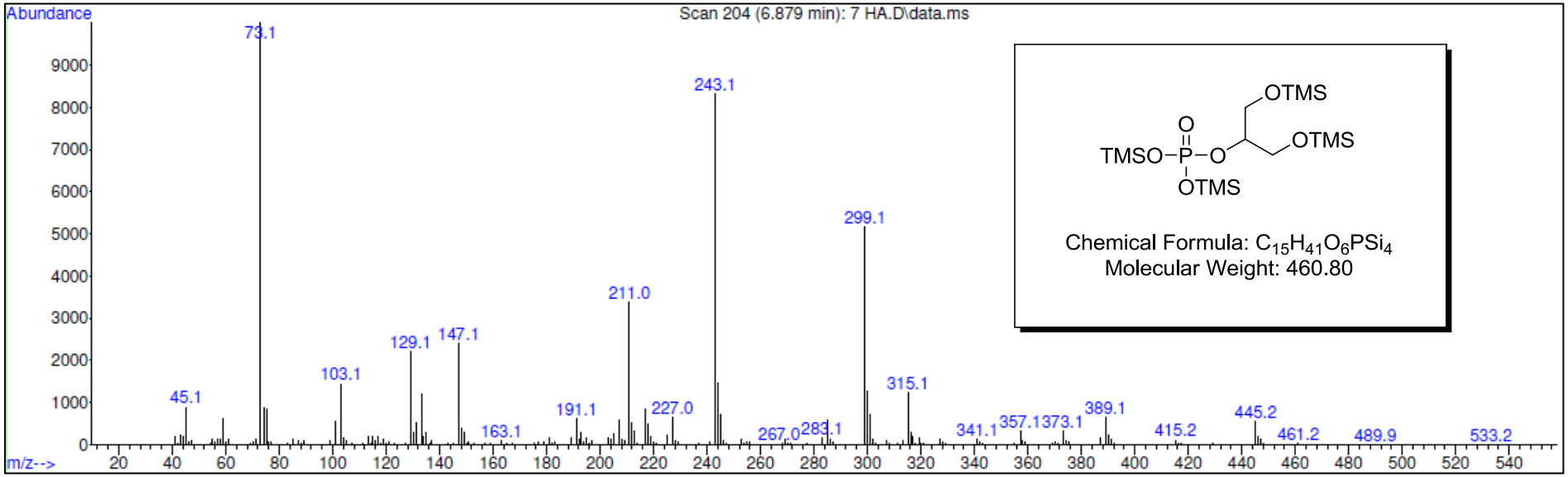



Figure C8: Mass spectroscopic analysis of the molecule that emerged at 6.848 min on figure C6. This peak contains phosphate groups, most likely from the relatively non-polar phospholipids of the cell membrane.